Motivational memory: role of nicotinic receptors in synaptic plasticity

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Award date:
2019

Awarding institution:
University of Bath

Motivational memory: role of nicotinic receptors in synaptic plasticity

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A thesis submitted for the degree of Doctor of Philosophy

University of Bath

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September 2018

ACKNOWLEDGEMENTS

This work was performed under the supervision of Dr. Chris Bailey, Prof. Sue Wonnacott and Prof. David Heal with financial support from a BBSRC SWBio CASE studentship with RenaSci, Nottingham, UK. My special thanks go to my supervisors who have supported me in various ways throughout my whole PhD. Thanks to Sue (and Dora!) for giving me somewhere to live while I was in between homes. For technical support at Bath, my thanks go to the 4S annexe staff, in particular Martin, Jane, Lesley and Jean for their infinite patience, training and welcome chats while spending lonely days in the experimental room.

I would like to thank Prof. David Heal for giving me the opportunity to learn heroin IVSA at RenaSci. Many thanks to Sharon, Elaine, Michelle and Mike and all RenaSci staff for all their support and to Ian for putting me up for my stay in Nottingham. It was a thoroughly enjoyable and valuable experience which shaped my PhD for the better.

There are too many people to acknowledge in the PhD office, including past students, but to all, thanks for making this PhD more endurable in some way. From random chats in the office, trips to the pub, (finally!) playing badminton with me (let it be known I beat Emma once); to making me have a dishwasher loading song, it all helped me keep my sanity.

I am eternally grateful to my wonderful parents Karen and Thierry for giving me all the opportunities and support to achieve my ambitions; to my sisters Jessica, Victoria, Laura and my brother-in-law, Curran for providing distractions and places to visit on weekends. I could not have done it without all your support throughout the years. I thank Nenad for being by my side through it all, for being my best friend, and for helping me realise my dream of moving to Australia.

ABSTRACT

Drug addiction is a chronically relapsing disorder characterised by compulsive and uncontrollable drug consumption despite negative consequences. It is considered that in addiction, associative memories are formed, which trigger cravings and prompt relapse during abstinence, which is the largest unmet need of current addiction treatments. Nicotinic acetylcholine receptors (nAChRs) have been implicated in modulating addiction behaviours with drugs of abuse other than nicotine, and studies have provided evidence for a role of α7nAChRs in a morphine reward-based learning paradigm, conditioned place preference (CPP). The aim of this thesis was to characterise the role of α7nAChRs in motivational learning using a more potent opioid, heroin. The selective α7nAChR antagonist methyllycaconitine (MLA) was used to determine if these receptors contribute to the rewarding effects of heroin, the acquisition or the reinstatement of heroin CPP and heroin intravenous self-administration (IVSA). In 6-7 week old male Wistar rats, MLA had no effect on the primary rewarding effects of heroin in either CPP or IVSA experiments. When administered prior to heroin-primed reinstatement of CPP, MLA significantly inhibited reinstatement, demonstrating a selective effect of α7nAChRs on the reinstatement of CPP. Its effects on the heroin- and cue-primed reinstatement of IVSA were however inconclusive due to large variability. The molecular mechanisms of this effect were investigated by immunohistochemistry in brain slices of rats treated with either saline or MLA prior to heroin-primed reinstatement of CPP. AMPA GluA1 and phosphorylated GluA1 subunits were quantified by near-infrared scanning or confocal microscopy but no changes were detected due to methodological issues. The possible mechanistic actions of α7nAChRs in the relevant brain circuits are discussed.

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ABBREVIATIONS

5-HT 5-hydroxytryptamine

6-OHDA 6-hydroxydopamine

ACh Acetylcholine

AMPA 2-Amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid

ANOVA Analysis of variance

BF Basal Forebrain

BLA Basolateral amygdala

BNSTv/mp ventral/posteromedial bed nucleus of the stria terminalis

BSA Bovine serum albumin

CA1-3 Cornu ammonis regions 1-3

CAMKII Calcium/calmodulin-dependent protein kinase II

cAMP cyclic adenosine monophosphate

CEA Central nucleus of the amygdala

CNQX cyanquixaline (6-cyano-7-nitroquinoxaline-2,3-dione)

CNS Central nervous system

CPP Conditioned place preference

CPu Caudate putamen

CREB cAMP response element-binding protein

CS Conditioned stimulus

DA Dopamine

DAMGO ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin)

DAPI (4′,6-diamidino-2-phenylindole)

DG Dentate gyrus

dHPC Dorsal hippocampus

DHβE Dihydro-beta-erythroidine

DR Dorsal raphe

EC Entorhinal cortex

ECL Enhanced chemiluminescence

EPSC Excitatory postsynaptic current

ER Endoplasmic reticulum

FC Frontal cortex

FDA Food and drugs administration

FR Fixed ratio

GABA Gamma-aminobutyric acid

GT Goal-tracking phenotype

HPC Hippocampus

i.p. intraperitoneal

i.v. intravenous

ICj Islands of Calleja

IL Infralimbic

IPN interpeduncular nucleus

IVSA Intravenous self-administration

KMO kynurenine 3-monooxygenase

LC Locus coeruleus

L-**DOPA** L-3,4-dihydroxyphenylalanine

LDT Lateral dorsal tegmental nuclei

LH Lateral hypothalamus

LTP Long term potentiation

MAGUK Membrane-associated guanylate kinase

MK-801 Dizocilpine

MLA Methyllycaconitine

mPFC Medial prefrontal cortex

MS medial septum

mTOR Mechanistic target of rapamycin

NAc Nucleus accumbens

nAChR Nicotinic acetylcholine receptor

NAM Negative allosteric modulator

NBM Nucleus basalis of Meynert

NMDA N-methyl-D-aspartate (receptor)

PAM Positive allosteric modulator

PAZ Presynaptic active zone

PBS Phosphate buffered saline

PCC Pearson's correlation coefficient

PFA Paraformaldehyde

PFC Prefrontal cortex

PKA Protein kinase A

PKC Protein kinase C

PPT Pedunculopontine tegmental nuclei

PR Progressive ratio

PrL Prelimbic

PSD Postsynaptic density

PVDF Polyvinylidene difluoride

REM Rapid eye movement

ROI Region of interest

s.c Subcutaneous

SEM Standard error of the mean

SI Substantia innominata

SN substantia nigra

SNARE Soluble **NSF** attachment protein

ST sign-tracker phenotype

TBS Tris-buffered saline

TBST Tris-buffered saline-Tween

THC Tetrahydrocannabinol

US Unconditioned Stimulus

v/hDB vertical/horizontal limb of the diagonal band of Broca

vHPC Ventral hippocampus

VP Ventral pallidum

VTA Ventral tegmental area

CHAPTER 1 INTRODUCTION

1.1. Opioid addiction

1.1.1. Prevalence and social impact

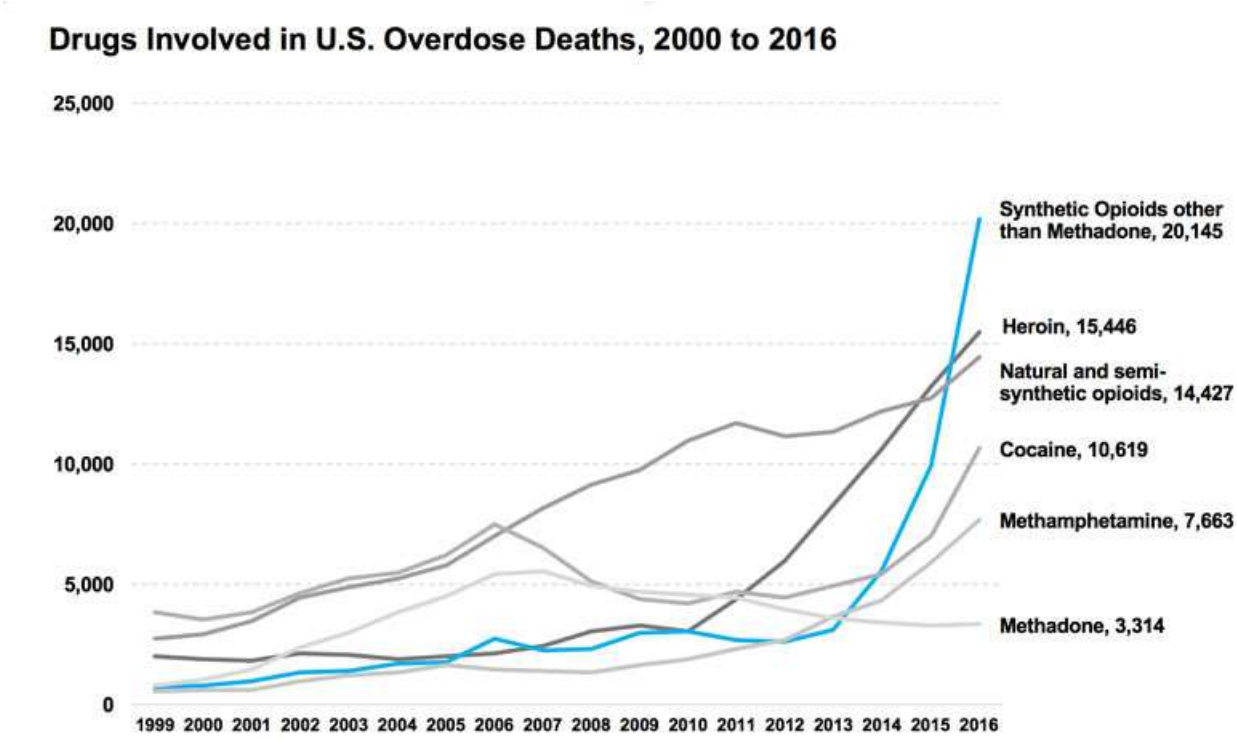


Figure 1.1: Opioid drugs involved in overdose deaths in the USA from 2000 to 2016 (NIDA, 2016).

Drug use and addiction are major public health issues, as it has been estimated that 3.5-7% of the world's population aged 15-64 tried an illicit drug at least once in 2012, and approximately 33 million drug users worldwide abused opioid drugs (UNODC, 2014). The number of deaths related to heroin abuse in the USA has risen greatly since 2010 and there is a more recent spike in deaths related to synthetic opioid abuse, which includes fentanyl, the highly potent heroin analogue (see Figure 1.1). In the UK in 2015, it was reported there were approximately 60 cases/million population of opioid-related overdose deaths, and 42% of all drug

addicts entered into treatment programmes were heroin users (European Monitoring Centre for Drugs and Drug Addiction, 2018).

A major part of the opioid abuse problem occurs from the abuse of prescription opioids before transitioning to illicit drugs (Centers for Disease Control and Prevention, 2017). In 2015, it was reported that over 12 million people aged 12 or over declared abusing an opioid prescription pain relief medication in the USA (Centers for Disease Control and Prevention, 2017). The rate of prescriptions of opioids increased greatly between 2000 and 2012; they have recently begun to decrease but still remain high (66.5 per 100 people in 2016 in the USA, Centers for Disease Control and Prevention (2017)).

Currently, therapies to treat opioid addiction involve either replacement therapy, using alternative μ opioid agonists such as methadone and buprenorphine; or with the μ and κ opioid receptor antagonist naltrexone, or behavioural therapy (for review, see Bart (2012)). Use of psychosocial treatments alone has a limited success rate, with 80% of patients relapsing. Opioid replacement with methadone and buprenorphine are used to prevent the negative affective and physiological state associated with withdrawal; and the use of naltrexone reduces the rewarding effects of opioids in addicts, however benefits diminish with time and again, the majority of patients undergoing this form treatment relapse (Bart, 2012). A major failure of these current therapies is cravings and relapse, and there is currently no treatment which is able to prevent this. It is therefore essential to study the brain circuitry and molecular mechanisms that underlie craving and relapse in order to develop more efficient pharmacotherapies.

1.1.2. Addiction Circuitry

Addiction is a chronic relapsing neuropsychiatric disorder characterised by compulsive seeking and consumption of drugs (and other hedonic activities), and the loss of control over drug intake despite negative consequences (Koob, 2013). There are several stages in the development of addiction. The primary stages of the acquisition of addiction were widely accepted to be manifested by rapid increases in dopamine release in the mesocorticolimbic pathway in the brain, however the role of dopamine in reward has since shifted Nutt et al. (2015).Studies have shown that dopamine receptor antagonists and the ablation of mesolimbic or nigrostriatal dopamine neurons have no effect on the palatability of food in animals but rather affect the incentive motivational value of these rewards (Berridge and Robinson, 1998, Berridge and Kringelbach, 2015). .

PET studies in humans have shown that ketamine, alcohol and cannabis do not necessarily induce dopamine release in the striatum (Aalto et al., 2002, Yoder et al., 2007, Barkus et al., 2010). Furthermore, dopamine receptor antagonists have conflicting effects on opioid self-administration and conditioned place preference behaviours in rodents (Pettit et al., 1984, Hnasko et al., 2005, Ribeiro Do Couto et al., 2005, Bossert et al., 2007, Galaj et al., 2015, Assar et al., 2016), and in humans, heroin was shown to have no effect on striatal dopamine levels, despite producing a pronounced euphoric high (Daglish et al., 2008).

In humans, dopamine receptor antagonists have been shown to have no effect on the subjective ratings of drug pleasure (Brauer and De Wit, 1997). In a discrete trial runway task, where rats approach a box in response to an olfactory cue signalling the availability of food or heroin; haloperidol had no effect on operant runway performance. Interestingly, the following day, rats that received haloperidol

were significantly slower in approaching the box. This suggests that the motivational arousal of the animal on the day of receiving haloperidol was intact, but rather the memory of a degraded reward discouraged the animals to seek the reward.

Additional studies have also shown that dopamine plays a role in the anticipatory, preparatory, appetitive, or approach phases of motivated behaviour (Blackburn et al., 1987, Schultz et al., 1993, Berridge and Robinson, 1998, Apitz and Bunzeck, 2014). Furthermore, dopamine transmission is also important in aversive motivational states, as elevated extracellular dopamine levels have been reported in the nucleus accumbens and forebrain regions in response to foot-shock and other types of stressor (Davis et al., 1994, Guarraci et al., 2000, Fadok et al., 2009), suggesting it is more than a 'liking' signal.

As the drug-taking behaviour becomes associated with the pleasurable effects of the drug, it is repeated in a Pavlovian manner as non-pharmacological contextual factors such as environments, people or paraphernalia become associated with drug intake (Stewart, 2008). These factors are repeatedly associated with the rewarding effects of the drug; therefore through Pavlovian conditioning they become triggers for craving and relapse. Because these strong associations are formed and become salient cues for drug taking, it is now believed that addiction is more than just a reward deficit and involves a component of learning and memory (Hyman et al., 2006). This chapter will introduce the reward circuitry involved in the acquisition of addiction and will rationalise the concept of addiction being a learning and memory disorder. Additionally, the pathways involved in mediating this behaviour will be discussed.

1.1.3. The dopamine circuitry in addiction

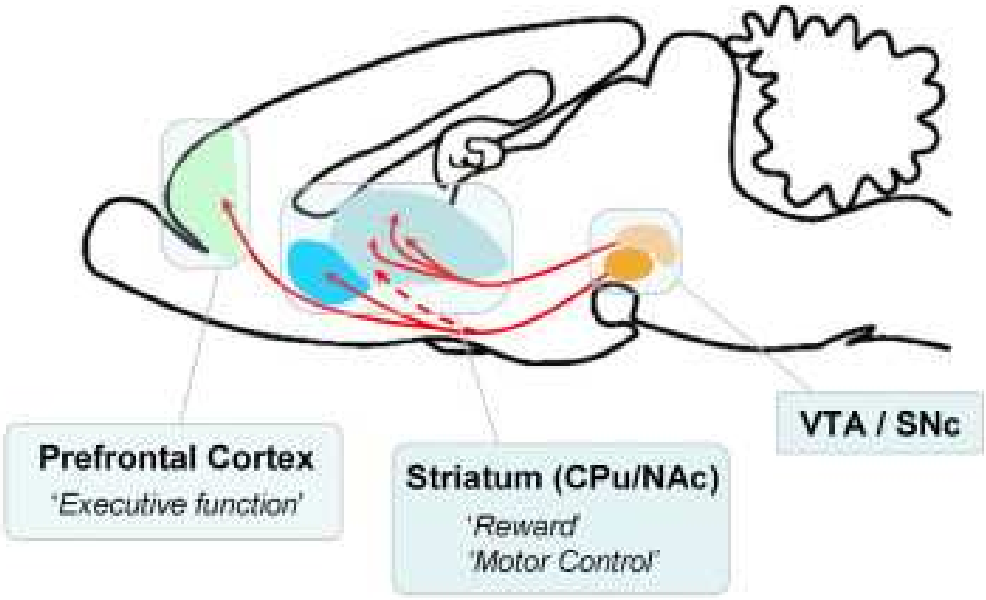


Figure 1.2: Diagram of the dopaminergic circuitry in the rodent brain. Dopaminergic neurons project from the substantia nigra compacta (SNc, light orange) to the caudate putamen (CPu) or dorsal striatum (light blue), which forms the nigrostriatal pathway. The ventral tegmental area (VTA, orange) projects to the nucleus accumbens (NAc, dark blue), which forms the mesolimbic pathway; and to the prefrontal cortex (light green), which forms the mesocortical pathway. To a lesser extent, the VTA also projects to the dorsal striatum (dotted line). Taken from (Livingstone and Wonnacott, 2009).

Most drugs of abuse increase dopaminergic transmission, by either directly targeting dopaminergic neurons in the ventral tegmental area (VTA) or increasing extracellular dopamine levels in the nucleus accumbens (NAc) or both (Van den Oever et al., 2010). The mesocorticolimbic dopaminergic pathway is composed of axons of dopaminergic neurons in the VTA that project to the NAc (see Figure 1.2 above), prefrontal cortex (PFC), but also to the amygdala and hippocampus (van Huijstee and Mansvelder, 2014). Dopaminergic neurons from the substantia nigra (SN) extend to the dorsal striatum to form the nigrostriatal pathway (Figure 1.2).

These midbrain dopamine neurons are tonically active but show phasic activation following primary food rewards or stimuli associated with the presentation of rewards (Schultz, 2013). The importance of these regions in reward seeking was shown by 6-OHDA lesions (Smith et al., 1985), microinfusions of dopamine antagonists (Bachtell et al., 2005) and dopamine D1 receptor knockout mice (Caine et al., 2007), as these interventions all reduced reward seeking.

Despite the vast research in dopamine and reward (Koob, 2013), the dopamine model of addiction is too simplistic. Studies have shown that although dopamine is important in the acquisition of hedonic behaviours, it is not essential for reward-based learning. Lesions of dopaminergic cells in the SN produced aphagia in rats but reactivity to aversive or palatable tastes remained normal, showing a dissociation of hedonic responses to dopamine (Berridge and Robinson, 1998). In another study, genetically engineered dopamine-deficient mice produced similar responses to control mice in the learning of salient cues and in responding to the hedonic effects of rewards (liking), but dopamine was necessary for the seeking of rewards in a T-maze model of goal oriented behaviour (wanting) (Robinson et al., 2005). In opioid models of reward-based learning, it was shown that dopamine was not required for the hedonic responses to opiate administration or for learning cues predictive of opioid administration, as dopamine-deficient mice were still able to acquire robust morphine conditioned place preference (CPP) (Hnasko et al., 2005). These dopamine-deficient mouse models however required intermittent L-DOPA treatment to survive, and due to locomotor deficiencies, required caffeine to perform tasks, therefore the results from these studies cannot be directly correlated to a lack of dopamine (Fields and Margolis, 2015). Other studies have however shown that lesions to the NAc in rats inhibit cocaine self-administration

but do not inhibit heroin self-administration (Pettit et al., 1984). These initial findings support the hypothesis that additional neural substrates than dopamine are required in addiction.

Dopamine has two main roles in reward processing, which are believed to be mediated by different pathways in the reward circuitry. The first role is linked to motor behaviour, to elicit behavioural responses previously paired with the stimulus, and is thought to be mediated by the mesolimbic circuit (Hyman et al., 2006). The second role is to inform the individual of the appearance of a novel salient stimulus to prompt neuromodulatory changes associated with learning and memory. This behaviour is thought to be mediated by the mesocortical pathway (VTA to medial PFC), as this pathway is involved in cognitive control and motivational responses.

1.1.4. Beyond the mesolimbic circuit

A common feature in addicts is that taking drugs becomes valued above all other goals and abstinence can prove very difficult as drug-associated cues and emotions can trigger feelings of craving (Van den Oever et al., 2010). This implies addiction is a learned behaviour involving plasticity. Many neuroimaging studies in humans have demonstrated a key role of the frontal cortex (FC) in compulsive behaviours and in the regulation of cognitive and emotional processes which results in the over-valuing of drug reinforcers over other rewards (Volkow et al., 1993, Volkow and Fowler, 2000, Goldstein and Volkow, 2002).

The prefrontal cortex (PFC) has been shown to be a major region involved in working memory, which is the ability to process information, update it with other

information and use it to guide behaviour (reviewed by Van den Oever et al. (2010)). Reversible pharmacological inhibition of the medial PFC (mPFC) has been shown to inhibit the reinstatement of drug seeking in self-administration paradigms (McLaughlin and See, 2003, Rogers et al., 2008). Dopaminergic modulation of plasticity in the PFC has been closely linked to reward-related learning, as NAc core (the inner part of the NAc) glutamate levels can be enhanced by direct injection of cocaine in the mPFC (Park et al., 2002), and can also be inhibited by pharmacological inactivation of mPFC projections to the NAc core (LaLumiere and Kalivas, 2008). This pharmacological inactivation of glutamate release in the NAc core also inhibits the reinstatement of drug seeking (LaLumiere and Kalivas, 2008), suggesting an important role of the PFC in modulating drug-seeking behaviour.

The mPFC also receives dopaminergic input from the VTA (Van den Oever et al., 2010, Sheynikhovich et al., 2013), which is believed to be involved in reinforcing working memory associated with reward. Intra-mPFC injections of dopamine receptor agonists have been shown to trigger reinstatement, whereas antagonists have been shown to attenuate this behaviour (McFarland and Kalivas, 2001, See, 2009). Furthermore, the context-induced reinstatement of heroin seeking has been shown to be associated with Fos induction in the mPFC as a marker of neuronal activation, and the selective inhibition of mPFC neurons has been shown to inhibit this reinstatement behaviour (Bossert et al., 2011). It has therefore been hypothesised that the PFC is involved the assignment of values to rewards and to gate goal-oriented behaviour. It has vast connections with many brain regions (see Figure 1.3), which places it in a prime position to integrate motivational information and drive drug-seeking behaviour. The dopaminergic input from the VTA to the

PFC posits a role of the PFC in reinforcement learning, and a distorted dopamine signal in this pathway has been hypothesised to produce the over-learning of drug-related cues (Van den Oever et al., 2010).

Although the mesocorticolimbic system is heavily implicated in the processing of natural and unnatural rewards, the activation of this pathway is not sufficient to explain drug-seeking behaviour and the persistence of this behaviour. Dopamine is not thought to drive drug-seeking behaviour, but rather to gate it (reviewed by Wise (2004) and Schultz (2013)); revealing that the role of dopamine release in the NAc is more subtle and complex than a mere consequence of drug intake.

1.1.5. GABAergic modulation

GABAergic signalling is also thought to be important in modulating motivational responses. The main projections from the NAc and dorsal striatum are GABAergic (see Figure 1.3) and it is thought that acute drug use decreases GABAergic output from the NAc shell, the outer region of the NAc (Carlezon and Thomas, 2009). Furthermore, VTA dopamine cells (Kalivas, 1993) and glutamatergic neurons in the hippocampus (Mann and Paulsen, 2007) and PFC (Banuelos and Woloszynowska-Fraser, 2017) modulated by GABAergic neurons. The importance of GABAergic projections has been emphasised, particularly in the genesis of theta rhythms in the medial septum and hippocampus (Mann and Paulsen, 2007). It is thought that GABA regulates overall circuit tone and all drugs of abuse have been shown to influence inhibitory synaptic plasticity (Fields and Margolis, 2015). The present study will however focus on glutamatergic synaptic plasticity.

1.1.6. Glutamatergic circuitry

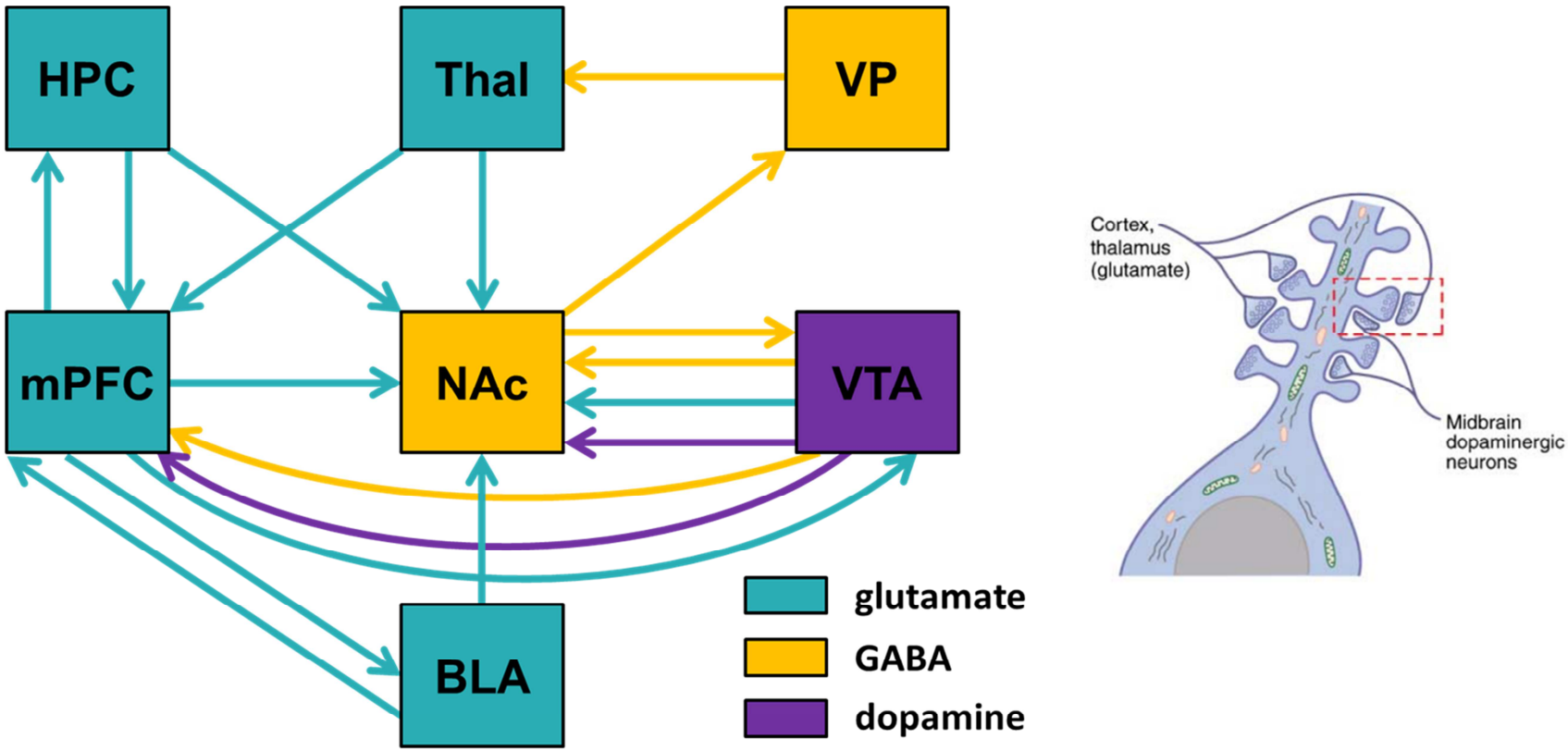


Figure 1.3: **Left:** Schematic diagram of the glutamatergic (blue), GABAergic (orange) and dopaminergic (purple) pathways involved in addiction-associated behaviour. HPC: hippocampus; Thal: thalamus; VP: ventral pallidum; FC: frontal cortex; NAc: nucleus accumbens; VTA: ventral tegmental area; BLA: basolateral amygdala. Summarised from (Luscher and Malenka, 2011, Madsen et al., 2012a). **Right**: Representation of converging glutamatergic and dopaminergic inputs onto striatal and other corticolimbic neurons (Kelley, 2004).

As previously discussed, dopamine is important for the reinforcement of a reward, but it cannot explain how repeated use of drugs abuse becomes compulsive, how drug-related cues can drive drug-seeking behaviour, or how the risk of relapse can persist for years after abstinence. The integration of dopamine and glutamate-coded signals at the molecular and cellular level in the NAc has been proposed as the drive behind addiction-related behaviour, and that synaptic plasticity is the substrate for these persistent changes in behaviour (Kauer and Malenka, 2007). As shown in Figure 1.3, there is extensive glutamatergic innervation of the NAc and the VTA, which converges with dopaminergic circuits. Furthermore, dopaminergic and glutamatergic terminals are localised in close proximity to each other on the same dendritic spines, demonstrating a potential for cellular plasticity involving glutamatergic and dopaminergic processes (Kelley, 2004). Studies have shown that drugs of abuse induce long-lasting changes in the brain, and although drugs of abuse alter many different synapses, they are thought to mainly alter glutamatergic transmission (Lüscher, 2013).

Changes in synaptic plasticity have been shown to occur after the first exposure to the drug of abuse. An original study showed that a single *in vivo* administration of cocaine to mice and rats resulted in an increase in ratios of AMPA/NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) in the VTA 24 hours after administration (Ungless et al., 2001). It was demonstrated that this increase in AMPA/NMDA ratio was due to an increase in AMPA receptor transmission. This cocaine-induced effect was found to be specific to dopaminergic cells in the VTA, since no effect was observed in GABAergic cells, or in the hippocampus (Ungless et al., 2001). Furthermore, this synaptic strengthening was found to be NMDA receptor-dependent, as co-administration of cocaine with an NMDA receptor

antagonist showed no changes in NMDA/AMPA ratios in the VTA. Since this study, it has been shown that all drugs of abuse (nicotine, morphine, ethanol, amphetamines and benzodiazepines) induce a potentiation of AMPA receptor transmission in dopaminergic cells in the VTA (for review, see van Huijstee and Mansvelder (2014)). Thus, a single exposure to drugs of abuse induces synaptic changes similar to NMDA-dependent long-term potentiation, which will be discussed further in section 1.2.2.

1.1.7. How does this relate to drug-related behaviour?

Establishing a causal relationship between drug-evoked synaptic plasticity and drug-related behaviour is challenging, in part because of the temporal discrepancy between the two phenomena. Non-contingent drug administration results in short-term synaptic plasticity in the VTA (van Huijstee and Mansvelder, 2014). These initial alterations in synaptic strength are the molecular underpinnings of circuit remodelling, which, with repetitive exposure, leads to behavioural alterations. *In vivo* models of drug self-administration allow contingent drug administration in animals, and synaptic potentiation in VTA dopaminergic neurons was shown to still be present 3 months after cocaine self-administration in rats (Chen et al., 2008), which suggests that synaptic strengthening in the VTA is an essential phenomenon in the development of addiction.

Mice with genetic deletions of the AMPA GluA1 (but not GluA2) or the NMDA GluN1 subunit selectively on dopaminergic neurons lacked drug-induced plasticity in the VTA (Engblom et al., 2008b); but the acquisition of significant conditioned place preference to cocaine was observed in these transgenic mice. Extinction

was however absent in the GluA1-lacking mice, and reinstatement was abolished in CPP, suggesting plasticity in additional brain regions other than the VTA is also important in the behavioural manifestation of addictive behaviours.

Unlike in the VTA, a single acute injection of a drug of abuse does not induce synaptic plasticity in the NAc (Kourrich et al., 2007). The NAc gates rewarding and aversive stimuli and directs goal-oriented behaviour. The principal neurons of the NAc (core and shell) are GABAergic medium spiny neurons (MSNs) which receive glutamatergic inputs from brain regions (see Figure 1.3) including the PFC and thalamus, which are involved in goal-oriented behaviour; the dorsal hippocampus, which likely provides spatial and contextual information about stimuli; the ventral hippocampus, and the amygdala, which contribute to a limbic circuit important for emotional processing of stimuli (Chartoff and Connery, 2014). Glutamate transmission is kept under tight control in the NAc. Glutamatergic afferents synapse on dendritic spines and these are modulated by extrasynaptic dopaminergic inputs (Figure 1.3, Chartoff and Connery (2014)). This allows the close regulation of excitability of these neurons by local release of dopamine, where $G_{\alpha s}$-coupled D1 dopamine receptors leads to the excitation of MSNs and $G_{\alpha i}$-coupled D2 receptors inhibit MSNs (Carlezon and Thomas, 2009). In general, aversive stimuli activate NAc neurons, which project to the VTA and ventral pallidum, whereas rewarding stimuli appear to inhibit NAc neuron firing.

Microinjections of AMPA into the NAc have been shown to elicit the reinstatement of cocaine seeking behaviour (Cornish and Kalivas, 2000), while AMPA receptor antagonists have been shown to inhibit reinstatement (Ribeiro Do Couto et al., 2005). Additionally, reduction of NMDA GluN2 subunit expression by siRNA injection into the NAc was shown to inhibit morphine CPP in rats (Kao et al.,

2011), demonstrating this is also present with opioid drugs. These findings demonstrate that glutamatergic synaptic plasticity is a key component of all stages of addiction, notably for the formation of drug-associated cues.

1.2. Addiction is a learning and memory disorder

As previously mentioned, the development of environmental contexts into salient cues for drug taking and the long-lasting risk of relapse cannot merely be explained by dopaminergic transmission in the mesocorticolimbic system (Koob, 2013). Rather, it has been long postulated that the different stages of addiction (acquisition, withdrawal and relapse) involve the hijacking of learning and memory systems in different brain regions for the encoding of persistent drug-related memories (Kelley, 2004, Hyman et al., 2006). It is therefore important to understand the molecular mechanisms underpinning memory encoding and how behavioural paradigms can be used to model these addiction-related memories.

1.2.1. LTP underlies memory processes

Figure 1.4: Schematic representation of the processes that occur following re-exposure to a drug associated cue. Brief cue exposure initiates LTP mechanisms which lead to memory reconsolidation and the strengthening of the drug-associated memory, which increases relapse potential. Figure adapted from (Rich and Torregrossa, 2018).

Memories are stored and can be retrieved in response to social and environmental cues, termed consolidation. They can also be reinforced when they are retrieved by the process termed reconsolidation, where the synapses involved are strengthened further by LTP (Figure 1.4, Rich and Torregrossa (2018)). Adrenergic receptor agonists were among the first ligands to be proposed to interfere with drug-cue memory reconsolidation as following the re-activation of fear-conditioned memories, treatment with the α1 agonist clonidine or the β adrenergic receptor antagonist propranolol disrupts memory reconsolidation in the context of post-traumatic stress disorder (Debiec et al., 2011, Gamache et al., 2012). Moreover, propranolol has been shown to disrupt the reconsolidation of cocaine and morphine-paired memories (Bernardi et al., 2006, Robinson and Franklin, 2007) and the inhibition of proteins involved in synaptic plasticity in neurons such as CAMKII, PKC, ERK and mTOR all inhibit the reconsolidation of drug-related memories (for review, see Rich and Torregrossa (2018)). Reconsolidation is the mechanism believed to underlie drug craving and seeking, triggered by stress, social and environmental cues associated with drug-taking, and exposure to even small doses of the drug (Bouton, 2002). Animal behavioural paradigms of drug-related learning and memory have been used to understand the causes of drug craving and seeking, and could potentially reveal new targets for better pharmacotherapies.

1.2.2. Long term potentiation as a substrate of learning and memory

One of the key molecular mechanisms underpinning memory processes is thought to be long term potentiation (LTP). Long-term depression (LTD) is also a key mechanism of memory processing, but this thesis will focus on the most extensively studied form of LTP, NMDA-dependent LTP (Figure 1.5, Kauer and Malenka (2007)).

LTP triggers the trafficking of further AMPA receptors to the surface to increase receptor density at the synapse (Figure 1.6). AMPA receptors are tetrameric ionotropic glutamate receptors that mediate fast excitatory transmission in the brain. There are four genetically distinct subunits of AMPA receptors (GluA1-4) and combinations of these form tetrameric receptors in the endoplasmic reticulum (ER) (Luscher and Malenka, 2012). In neurons of the rat hippocampus, AMPA receptors mainly contain either GluA1/2 or GluA2/3 subunits (Wenthold et al., 1996), with mainly the former present at the synapse (Lu et al., 2009). GluA4 subunits are rare in the adult rat brain and are mainly expressed in the developing brain (Henley and Wilkinson, 2013). The GluA2 subunit contains a modifiable glutamine residue which can be switched for an arginine residue in the channel lining (Greger et al., 2002). This switch functions as an ER retention motif and causes the GluA2-containing AMPA receptors to be impermeable to calcium (Wright and Vissel, 2012). Most GluA2 subunits in the adult brain contain this arginine switch, which regulates the permeability and channel conductance of the receptor. The GluA1 receptor does not express this motif and is thus permeable to calcium. Subunits possess either short or long intracellular C-terminal domains which determine their trafficking (Greger et al., 2017). Long-tailed subunits (GluA1

and GluA4) are trafficked rapidly from the ER to the synapse and predominate the trafficking of the short-tailed C-terminus containing subunits (GluA2 and GluA3), which is slower (Mah et al., 2005, Kessels and Malinow, 2009). Thus, if an AMPA receptor contains GluA1 and GluA2 subunits, it is trafficked rapidly from the ER. Studies have shown that calcium-permeable homomeric GluA1 AMPA receptors are rapidly trafficked to the synapse at the onset of LTP (Hayashi et al., 2000, Isaac et al., 2007, Greger et al., 2017) and then heteromeric calcium-impermeable GluA2/3 AMPA receptors slowly replace the homomeric receptors over hours to maintain LTP (Isaac et al., 2007). Studies have also shown GluA2/3-containing receptors are trafficked slowly (Mah et al., 2005) and replace existing AMPA receptors in the absence of LTP (Greger et al., 2017).

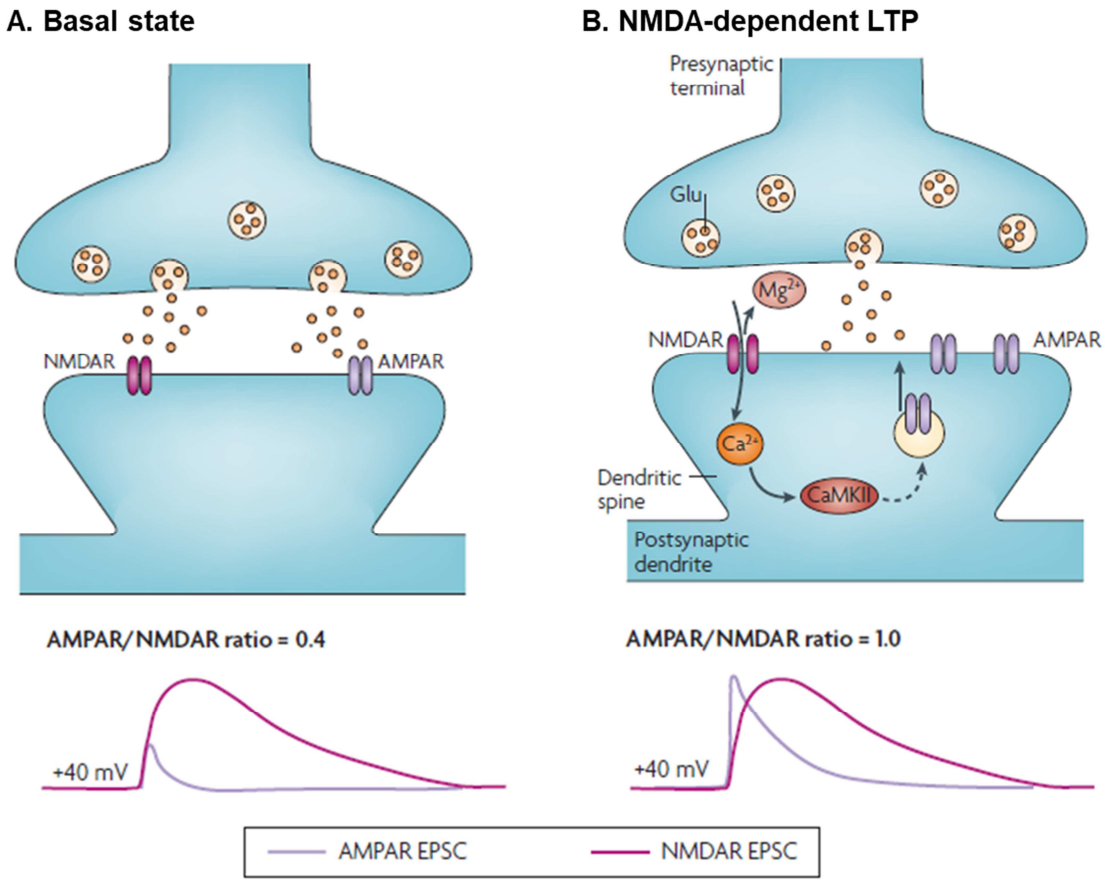


Figure 1.5: Schematic diagram of NMDA receptor (NMDAR, dark purple)-dependent long term potentiation (LTP) and representative AMPA/NMDA current ratios in the basal state (A) or during LTP (B). Strong and persistent release of glutamate (glu) from the presynaptic terminal (A) depolarises the postsynaptic terminal via AMPA receptors (AMPAR, light purple), which results in the relief of the voltage-dependent magnesium (Mg^{2+}) block from NMDA receptors (B) and the influx of calcium (Ca^{2+}) into the postsynaptic terminal. Intracellular calcium activates calcium/calmodulin-dependent protein kinases (CAMKII), which, though downstream mechanisms results in the insertion of AMPAR into the postsynaptic membrane, resulting in an increase in AMPA receptor density at the synapse, which can be measured by an increase in AMPA/NMDA ratios. This results in the strengthening of the synapse. Adapted from Kauer and Malenka (2007).

A diagram of the mechanisms is shown in Figure 1.5, where persistent release of glutamate from the presynaptic terminal results in a large influx of Ca^{2+} into the postsynaptic terminal. The resulting large increase in intracellular Ca^{2+} concentration activates a calcium/calmodulin-dependent protein kinase II (CAMKII), which triggers the insertion of further AMPA receptors to the synapse.

CAMKII undergoes autophosphorylation following induction of LTP and is essential in AMPAR exocytosis. CAMKII knock-out studies (Silva et al., 1992, Hayashi et al., 2000) and removal of the autophosphorylation site (Giese et al., 1998, Chang et al., 2017) showed a lack of LTP induction, suggesting a major role of CAMKII in this process.

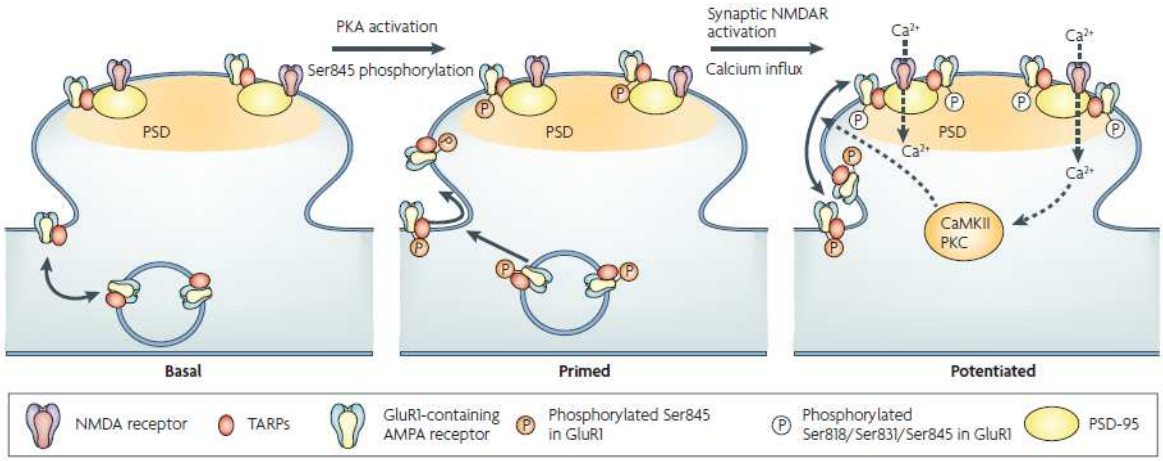


Figure 1.6: Proposed steps of the induction of LTP involving the trafficking of AMPA receptors to synapses. In basal conditions (left), AMPA receptors routinely cycle to and from the synapse by transmembrane AMPA receptor regulatory proteins (TARPs). Middle: the activation of PKA by depolarisation of the postsynaptic terminal results in the phosphorylation of GluA1-containing AMPA receptors into perisynaptic sites, therefore dendritic spines are deemed primed for LTP. Right: NMDA receptor activation results in the increase in calcium influx, which triggers the trafficking of perisynaptic AMPA receptors to the synapse via CAMKII (and potentially PKC) signalling. AMPA receptors are stabilised in the postsynaptic membrane by the scaffolding protein PSD-95. GluA1 subunits are phosphorylated at serine 831 to increase channel conductance and at 845 to increase channel open probability for the potentiation of synaptic strength. Figure taken from Derkach et al. (2007).

The development of LTP also involves phosphorylation of AMPA receptor subunits (Soderling and Derkach, 2000). The phosphorylation of GluA1 receptors at serine 845 by phosphate kinase A (PKA) in the intracellular C terminus of GluA1 subunits

results in an increase in the channel open probability (Banke et al., 2000). Importantly, phosphorylation of serine 845 also regulates the surface expression of AMPA receptor expression by the delivery of intracellular stores of GluA1 AMPA receptors to perisynaptic sites (illustrated in Figure 1.6), coincident with synaptic activity (Oh et al., 2006). Increasing the extrasynaptic pool of AMPA receptors was shown to result in stronger theta burst LTP in rat hippocampal slices and in cultured rat hippocampal neurons (Oh et al., 2006); demonstrating that the phosphorylation of GluA1 at serine 845 primes AMPA receptors for the induction of LTP. CAMKII phosphorylation of serine 831 in results in increased channel conductance in homomeric GluA1 receptors (Benke et al., 1998). Another study found there is no increase in conductance of GluA1/2 heteromeric AMPA receptors following phosphorylation by CAMKII (Oh and Derkach, 2005), thus GluA2 plays an inhibitory role in CAMKII phosphorylation.

This dynamic exchange of AMPA receptors at the synapse drives either synaptic strengthening or synaptic weakening, processes which underlie memory encoding and retrieval. *In vivo* models have been used to model learning and memory tasks, and these models can be used in the context of addiction-related behaviours.

1.3. *In vivo* models to study drug-related memory

The two most commonly used behavioural paradigms for studying drug-related behaviour and memories are conditioned place preference (CPP), a Pavlovian conditioning model of learning and memory, and self-administration, an operant correlate of learning and memory. Both of these models were used in this thesis (Chapters 2 and 3). Both paradigms can measure associative learning by classical

conditioning where a conditioned stimulus (CS, originally neutral stimulus) is presented with an unconditioned stimulus (US, an innately rewarding stimulus) sequentially, so that the animal learns to associate the CS with the US and consequently the probability of the behaviour is increased (Tzschentke, 2007, Panlilio and Goldberg, 2007).

1.3.1. Conditioned Place Preference

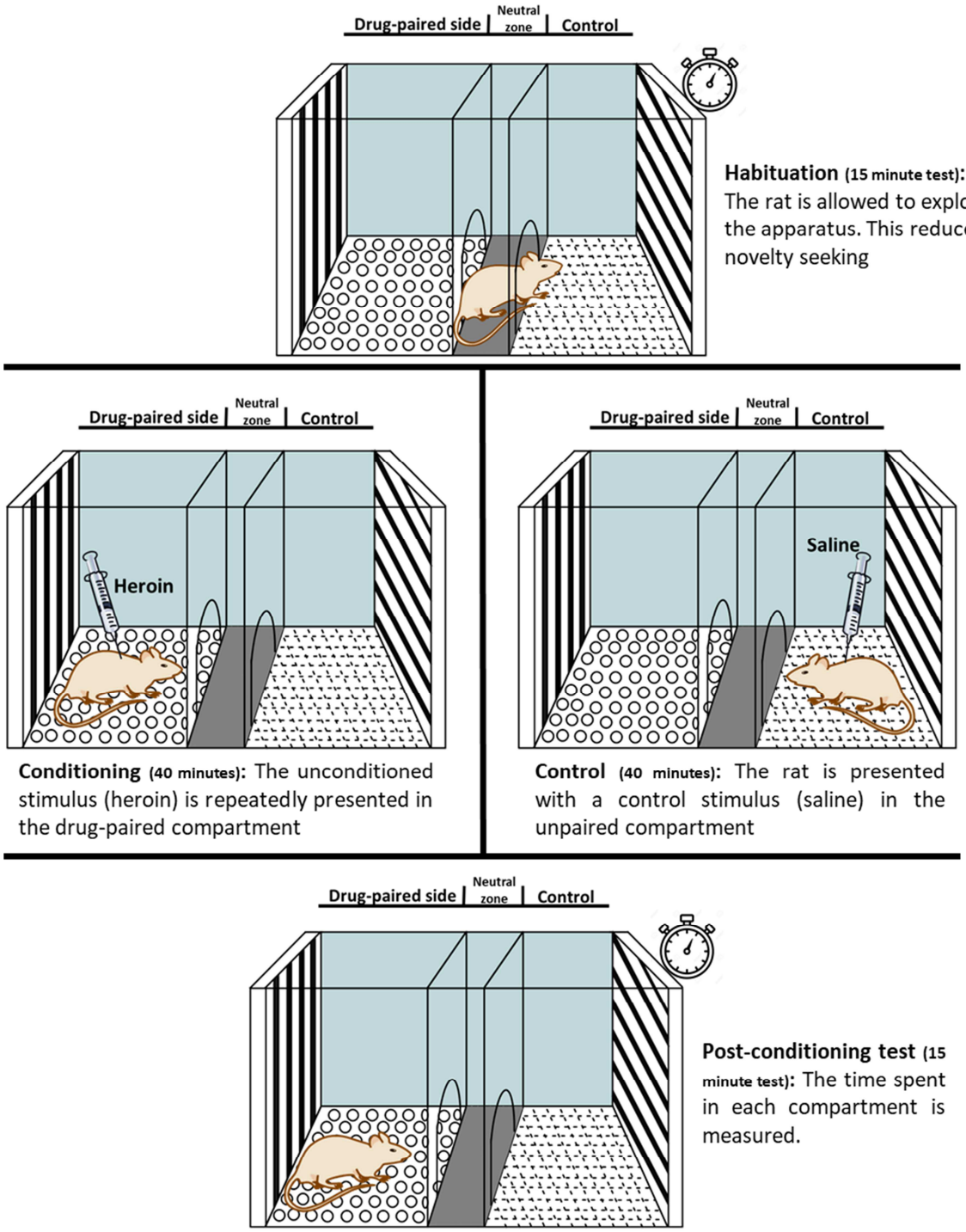


Figure 1.7: Schematic diagram of the habituation and conditioning stages of the CPP paradigm

The conditioned place preference (CPP) paradigm is used to study motivational memory associated with a rewarding or aversive stimulus (Cunningham et al.,

2006). The apparatus is generally divided into two compartments, differentiated by different patterns on the walls and different textured floors, and an optional third central neutral compartment (Figure 1.7). The neutral value of the compartments is measured during one or more (in this study, two) initial habituation sessions in which the time spent in either side is recorded before conditioning, and a baseline preference is established for each animal. This session also reduces the effect of novelty seeking. The next stage of the CPP protocol is the conditioning stage, where the conditioned stimulus, here, a CPP compartment, is repeatedly paired with an unconditioned stimulus (such as a heroin injection), so the association of reward from the US is formed with the CS (Figure 1.7). The opposite compartment is paired with vehicle and has no salient value for the animal. A post-conditioning test records the amount of time spent in either compartment in the absence of heroin (for a comprehensive review on the methodology, see Tzschentke (2007)).

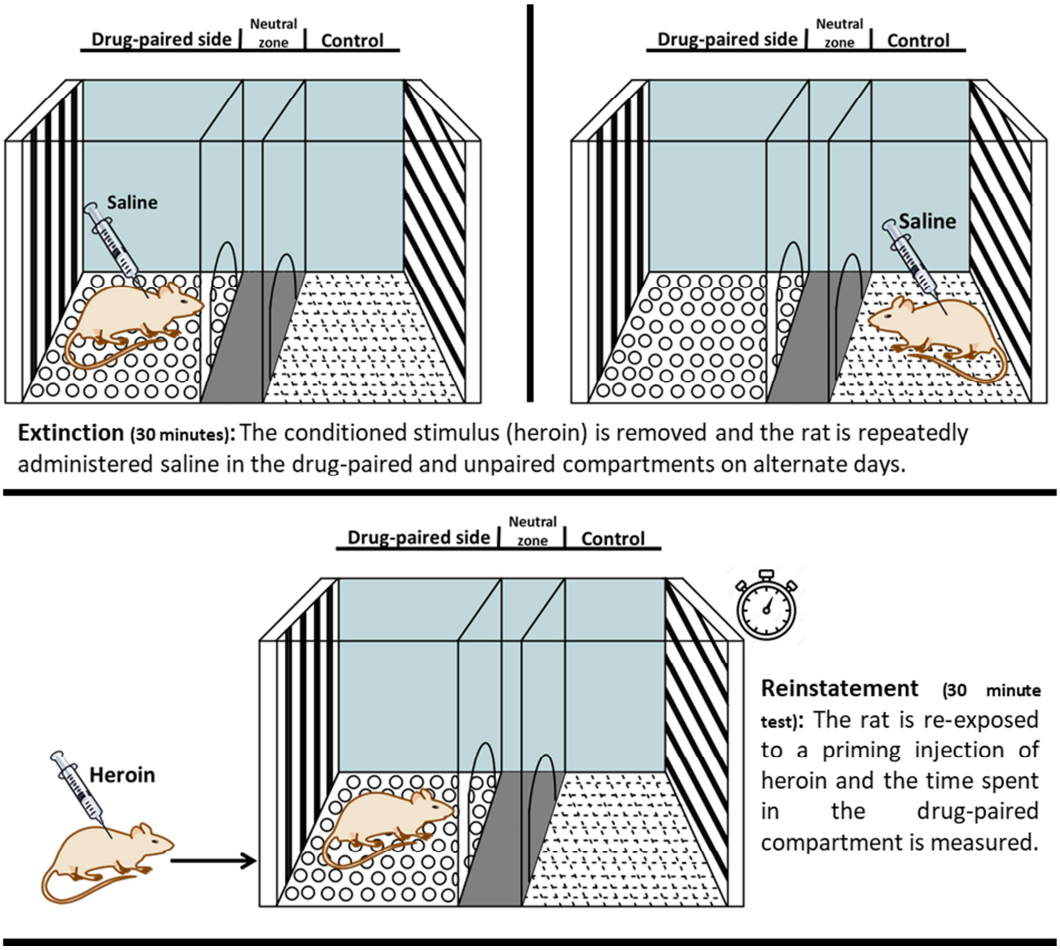


Figure 1.8: Schematic diagram of the extinction and reinstatement stages of CPP.

The next stage of CPP is the extinction phase. Extinction is defined as the disappearance of the conditioned behavioural response after the removal of the unconditioned stimulus that has reinforced the learning (Cunningham et al., 2006). This is usually done by re-exposing the animal to the previously drug-paired context in a drug-free state; either by administering repeated injections of vehicle in both the previously drug-paired and unpaired compartments, or by repeating CPP tests until the preference is no longer observed (**Figure 1.8**, Aguilar et al. (2009)). In the present study, the former method of repeated vehicle administrations in both compartments was used. A post-extinction test then records the time spent in the drug-paired side and the CPP (Figure 1.8) is

considered extinguished when there is no significant difference compared to the time spent in the drug-paired side during habituation.

The reinstatement stage of CPP is often used as an animal correlate of cravings and a return to drug seeking in studies investigating addiction treatments (Bossert et al., 2013). The extinguished drug-associated behaviour has not erased the drug-related memory, but it has been suppressed (Bouton, 2002). Reinstatement therefore refers to the retrieval of the conditioned behaviour in response to a cue. In the case of CPP, the cue is often the non-contingent exposure of animals to a priming dose of the drug, but can also be induced by other drugs of abuse or stress (Bouton, 2002). In the present study, reinstatement was induced by the re-exposure to heroin. The conditioned behaviour is deemed reinstated when there is a significant difference in the time spent in the previously drug-paired side compared to the post-extinction test (Aguilar et al., 2009).

1.3.2. Intravenous self-administration

Intravenous self-administration is a comprehensive behavioural paradigm which can model multiple complex aspects of motivational behaviour towards a drug reward. It is often used to predict the abuse liability of drugs and to study relapse-like behaviour (reviewed by Panlilio and Goldberg (2007)). In this model, animals are trained to perform an operant conditioned response (in this study, lever press) for a drug reward. Other types of reward such as food can be used but this study will focus on drug reinforcement. The motivational value of the drug can be determined by increasing the number of lever presses required to receive the reward (i.e. on a fixed ratio or progressive ratio), and the drug infusion is usually

paired with a contingent cue (such as a tone or light), which is presented simultaneously with the drug (Davis and Smith, 1976, See et al., 1999, Perry et al., 2014).

1.3.3. Schedules of reinforcement

The amount of work that has to be done to obtain the reward can be altered in self-administration studies. These are termed ratio schedules, which determine the number of responses required to obtain the reward (see Panlilio and Goldberg (2007) for review). The requirement can be constant (fixed ratio) or variable depending on the reward. The aim of these schedules is to maintain a characteristic pattern of responding, which are used as correlates of different aspects of addiction-related behaviour (Panlilio and Goldberg, 2007). Fixed ratio schedules are often used to determine the abuse liability of a drug, and the fixed ratio schedule was used in this thesis. Another type of schedule is the progressive ratio schedule, which assesses the effectiveness of a reinforcer, as under this schedule, the number of responses required for drug delivery progressively increases with repeated injections, until the subject ceases to respond for a certain amount of time (Panlilio and Goldberg, 2007). The progressive ratio therefore measures the motivational strength of the tested drug, and the highest response requirement that is achieved by the subject is termed the breakpoint.

There are other methods to determine the salient value of drug reinforcers. The unit price of the drug can be manipulated so the number of responses can determine the dose of drug to be received. For example, one lever press can result in a low dose of drug reinforcer, whereas three presses could result in a

higher dose, and the animal decides on the response. Choice can also be introduced into the paradigm, where an alternative reinforcer (food or a different drug of abuse) can also be an option for the subject to determine the value of the studied drug. Other types of schedule include second-order, chained, and multiple schedules (Panlilio and Goldberg, 2007), demonstrating the variety of parameters that can be modified to study a specific type of addiction-related behaviour.

Once self-administration is stabilised, the conditioned behaviour can be extinguished by the replacement of the drug with saline or by being placed in the operant chamber without being attached to the infusion pumps in the absence of the conditioned cues (Panlilio and Goldberg, 2007). In the present study, saline infusions were used to extinguish lever responses. Reinstatement procedures are used as a correlate of relapsing behaviour following a period of abstinence, where exposure to the previously drug-paired associations reinstates the previously extinguished conditioned behaviour (Davis and Smith, 1976, Perry et al., 2014). Three major types of treatment have been found to induce reinstatement of drug seeking in this paradigm, and each of these is analogous to the events that can trigger relapse in humans (Bouton, 2002). The first is drug priming by the re-exposure to the drug reinforcer or a different drug of abuse; the second is exposure to drug-associated cues and the third is stress (usually induced by foot-shock in self-administration studies) (Panlilio and Goldberg, 2007).

Drug self-administration is a highly adaptable technique for modelling various aspects of drug-related behaviour. Studies using this paradigm have elucidated key brain regions involved in the reinstatement of drug seeking, and interestingly, have determined that, though there are common regions involved in drug and cue-

primed reinstatement, there also appear to be distinct regions which respond to either type of stimulus to produce reinstatement.

1.3.4. Mechanisms of opioid relapse

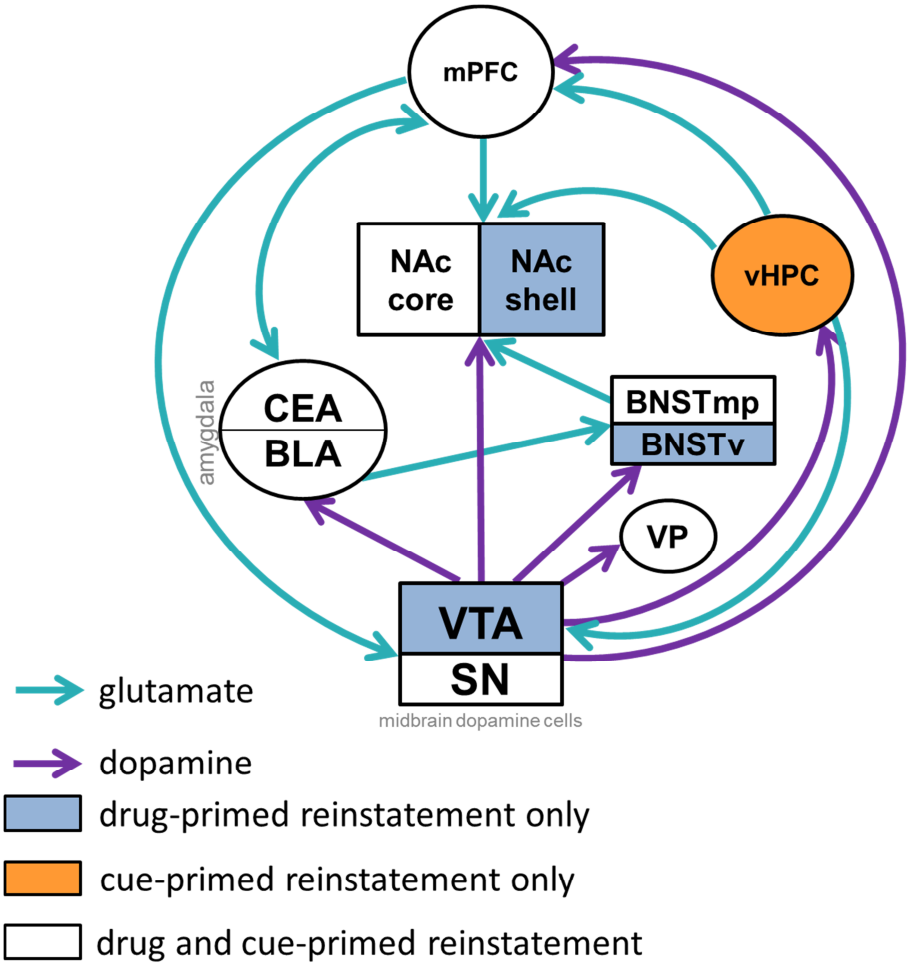


Figure 1.9: Proposed circuitry mediating relapse to heroin seeking. The only brain region to be reported to be involved exclusively in cue-induced reinstatement was the hippocampus (HPC, orange) (Namba et al., 2018). Light blue areas represent neural regions necessary for drug-primed reinstatement only and white areas represent neural regions involved in both cue- and drug-primed reinstatement of opioid seeking. mPFC: medial prefrontal cortex; NAc core/shell: nucleus accumbens core/shell; CEA: central nucleus of the amygdala; BLA: basolateral amygdala; BNSTmp: posteromedial bed nucleus of the stria terminalis; BNSTv: ventral bed nucleus of the stria terminalis; VP: ventral pallidum; VTA: ventral tegmental area. Purple arrows indicate dopaminergic signalling and teal arrows indicate glutamatergic signalling. Summarised from Rogers et al. (2008), Stewart (2008), Brown and Lawrence (2009) and Namba et al. (2018).

The previous sections have discussed the synaptic plasticity events in brain regions involved in the development of addiction. Relapse has been shown to involve the recall of drug-related memories, often brought about by the presentation of a drug-associated cue (Bouton, 2002). There are many *in vivo* studies investigating the roles of different brain regions involved in rodent models of relapsing behaviour with cocaine. Cocaine self-administration studies reported that reversible inactivation of the dorsal (not ventral) mPFC, nucleus accumbens core (but not shell) and ventral hippocampus decreased the reinstatement of cue-induced reinstatement of cocaine seeking (for review, see Bossert et al. (2013)). Brown et al. (2010) identified the brain regions that became activated in rats that reinstated drug-seeking behaviour in response to a cocaine prime in CPP, by the quantification of fos-positive cells (a marker of neuronal activation). They identified the PFC, BNST and nuclei (paraventricular and lateroanterior) of the hypothalamus, which were all shown to be involved in the reinstatement of cocaine in self-administration studies (Brown et al., 2010). They also showed activation of the lateral habenula, which receives inputs from the PFC, hypothalamus and basal ganglia (Baker et al., 2015); and is thought to drive behavioural flexibility. The activation of the lateral habenula has not previously been shown in operant studies, suggesting that there could be a distinction in the activation of certain brain regions in either CPP of self-administration studies.

The brain regions discussed above were thought to be common regions involved in reinstatement paradigms with all drugs of abuse; therefore there are few reports of brain regions involved specifically in opioid relapse. A study by Rogers et al. (2008) however identified additional regions such as the NAc shell, BNST, ventral mPFC, substantia nigra and ventral pallidum, which were involved in both the cue-

and drug-primed reinstatement of heroin seeking in heroin self-administration (see Figure 1.9). Furthermore, another study by Madsen et al. (2012b) compared brain regions involved in cue-induced reinstatement of either sucrose or morphine self-administration after protracted abstinence in mice. They found a multitude of brain regions activated with both rewards (including NAc shell, BNST, VTA, hippocampus, LC and lateral habenula), but the structures which were more activated in morphine seeking included the NAc core, BLA, SN pars reticulata and the CEA. These findings support a cortico-striatal limbic circuit activated during the reinstatement of opioid seeking, supporting previous findings.

1.3.4.1. Cue-induced reinstatement

Wikler (1973) and O'Brien et al. (1992) discovered that environmental contexts associated with drug taking could trigger relapse in humans. An initial study conducted by Davis and Smith (1976) showed that pairing a neutral cue (buzzer) with morphine intravenous infusions in rats produced robust lever pressing responses. Subsequent extinction replacing morphine with saline infusions in the absence of the cue reduced lever pressing. It was found that the presentation of the buzzer cue following extinction could re-trigger lever responses, demonstrating for the first time the role of cues in the reinstatement of drug self-administration and addiction. Grimm et al. (2000) demonstrated that presenting cues contingently during conditioning is essential for the development of drug reinforcement, and See et al. (1999) showed that the use of compound cues (tone + light) over using cues individually is even more effective. It has been shown that lever responses for cocaine or heroin in response to either a light and tone cue were similar in magnitude to each other (Panlilio et al., 1996, Panlilio et al., 2000). When the

compound tone and light cues were presented, there was a three-fold increase in responding for the lever compared to either cue alone under extinction conditions (saline infusions) and two-fold under maintenance conditions (heroin infusions) (Panlilio et al., 2000), demonstrating a strong effect of compound cues on drug seeking behaviour.

Since then, a multitude of studies have shown the influence of cues in extinction and reinstatement in preclinical models of relapse with many drugs of abuse such as heroin, speedball (cocaine and heroin mix), alcohol and nicotine (reviewed by Perry et al. (2014)). These cues are putative correlates of the contextual cues formed when addicts repeatedly take drugs, which trigger cravings and prompt relapse. To a certain extent, these cues have good construct validity for cue-reactivity in humans, however cue-induced reinstatement is perhaps less representative of human behaviour. This is mainly due to the fact that animals undergo extinction training, which is not apparent in most humans, and no studies have investigated the possible consequences on the predictive validity of the behavioural model. There is still much to be discovered in the neuronal substrates involved in both animal reinstatement and human relapse, but what is currently known will be discussed below.

See (2009) showed that antagonism of D1 dopamine receptors in the mPFC inhibited cue- and drug-primed reinstatement of heroin IVSA, demonstrating a role for dopaminergic signalling in in the PFC in cue-primed reinstatement. Interestingly, Shaham and Stewart (1996) found that dopamine receptor antagonists SCH23390 (D1 receptors), raclopride (D2) and haloperidol (D1 and D2) administered systemically blocked heroin-primed reinstatement of drug seeking in an intravenous self-administration paradigm, though this effect could be

due to an inhibition of the reinforcing effects of the heroin prime rather than an inhibition of the recall of the drug seeking behaviour. In the reinstatement of CPP, the role of dopamine becomes more controversial, since the reinstatement of CPP does not appear to be affected by DA receptor antagonists. Lesions of DA mesolimbic DA structures however block the reinstatement of CPP, adding confounding evidence to the role of dopamine. Ribeiro Do Couto et al. (2005) found that the dopamine receptor antagonists used in the study by Shaham and Stewart (1996) and the dopamine release inhibitor CGS10746B had no effect on the morphine-primed reinstatement of CPP in mice. The same study showed that the NMDA receptor antagonists memantine (20 and 40 mg/kg, i.p.) and MK-801 (0.2 and 0.3mg/kg, i.p.) blocked morphine-primed reinstatement of CPP in mice, demonstrating an essential role of glutamatergic signalling in the reinstatement of CPP. The route of administration was however systemic and the brain region involved was not determined. Wang et al. (2002) performed electrolytic lesions of different brain regions in rats and found that lesions in the VTA and NAc shell (but not core) inhibited morphine-primed reinstatement, suggesting a possible role of dopaminergic signalling in drug-primed reinstatement of CPP. However, the brain regions were totally ablated in these lesion studies, therefore the damaged processes could be glutamatergic or GABAergic in nature (see Figure 1.3); and a more selective lesion of dopaminergic neurons is needed to study this matter further. Furthermore, electrolytic lesions can also damage nerve fibres surrounding the lesioned region, therefore the damage to off-target nerve fibres could be a contributing factor to the lack of reinstatement.

Overall, there is a lack of definitive evidence implicating dopamine neurotransmission in the reinstatement of CPP with opiates. There are many more

studies which investigate and show a role of DA in cocaine reinstatement of CPP, therefore the lack of evidence with opiates could be explained by the generalisation of pathways involved in reinstatement for drugs of abuse. More recently, Assar et al. (2016) demonstrated that intracerebral infusions of SCH23390 (a D1 receptor antagonist) and sulpiride (a D2 receptor antagonist) into the CA1 of the dorsal hippocampus of male Wistar rats dose-dependently decreased the acquisition of morphine CPP. Infusions of SCH23390 before morphine-primed reinstatement of CPP dose-dependently attenuated the preference scores compared to saline-treated rats; however, these scores were still significantly different to the post-extinction test scores, suggesting the reinstatement of drug seeking behaviour was still occurring. With regards to the D2 receptor, only the highest dose of sulpiride significantly inhibited morphine-primed reinstatement of CPP (Assar et al., 2016). Combined together, these data suggest that these D1/D2 receptors antagonists reducing the salient value of the morphine rather than affecting the motivation of the animal to seek the reward.

Zhou et al. (2007) identified the role of cholinergic transmission in the NAc core by the administration of physostigmine (an acetylcholinesterase inhibitor), which inhibited cue-primed reinstatement of heroin IVSA. Context-induced reinstatement of heroin seeking was decreased by inhibiting glutamatergic transmission or dopamine receptors in the NAc shell but not core (Bossert et al., 2004, Bossert et al., 2007).

Portugal et al. (2014) showed that morphine-primed reinstatement of CPP in mice was associated with a significant increase in long term potentiation in the dorsal hippocampus. Furthermore, the local inhibition of GluN2B subunit-containing NMDA receptors in the dorsal hippocampus blocked morphine-primed

reinstatement of CPP in mice (Portugal et al., 2014). Wright et al. (2018) showed by autoradiography that morphine-primed reinstatement of CPP in rats induced a significant increase in [3H]-AMPA binding in the ventral hippocampus (**Figure 1.12**). These combined data indicate a strong link between glutamatergic synaptic plasticity in the hippocampus and morphine-primed reinstatement of CPP.

Another study found using Western blots that morphine-primed reinstatement of morphine CPP in rats significantly increased GluN2B NMDA subunit expression in the NAc and hippocampus, but saw no change in the PFC or the amygdala (Ma et al., 2007). Stress-induced reinstatement on the other hand, increased GluN2B expression in the NAc and amygdala, suggesting a difference in the neurochemistry underlying the different stimuli for reinstatement. They found no change in GluN1 subunit expression in these regions. Systemic administration of the GluN2B subunit-selective NMDA receptor antagonist ifenprodil (1-10 mg/kg, i.p.) dose-dependently attenuated morphine-primed reinstatement, and at the highest dose, significantly decreased CPP scores compared to vehicle-primed rats (Ma et al., 2007). There was no effect of ifenprodil on forced-swim stress-induced reinstatement, again reinforcing the notion of differences in the neurotransmitter pathways involved in different types of reinstatement in the same paradigm. Further investigation of the brain regions involved showed that microinjections of ifenprodil into the NAc shell and CA1 region of the dorsal hippocampus; once per day for 3 days after extinction and prior to reinstatement significantly blocked morphine-primed reinstatement of CPP (Ma et al., 2007). These data suggest that an up-regulation of GluN2B-containing NMDA receptors in the NAc is a common phenomenon in stress and drug-primed reinstatement of CPP; however the

increase in expression in the hippocampus is specific to drug-primed reinstatement of CPP.

The role of AMPA receptors in modulating motivational learning has been less extensively studied. Tzschentke and Schmidt (1997) found that the systemic administration of the non-selective AMPA receptor antagonist GYKI 52466 blocked the expression of morphine CPP in rats. Harris et al. (2004) showed that microinjections of the non-selective AMPA receptor antagonist CNQX into the VTA of rats inhibited the acquisition and expression of morphine CPP. At a molecular level, Billa et al. (2009) found that while the acquisition of morphine CPP in rats had no effect on AMPA receptor subunit expression in the hippocampus, saline extinction was associated with an increase in the phosphorylation of the GluA1 subunit at serine 845 in postsynaptic fractions. They found no difference in the expression levels of GluA1 or GluA2 subunits during extinction. They also reported that repeated unpaired injections of morphine decreased the expression of GluA1 and GluA2 subunits in postsynaptic fractions, suggesting an effect of morphine alone on AMPA receptor expression. In contrast, Moron et al. (2007) found that morphine administration increased GluA1 expression in the postsynaptic density in the mouse hippocampus (Moron et al., 2007). The above studies did not however observe the effect of inhibiting AMPA receptors or quantify AMPA receptor subunits after morphine-primed reinstatement of CPP.

It is important to bear in mind that AMPA and NMDA receptor antagonists have been shown to impair memory in a variety of learning and memory tasks (for review, see Riedel et al. (2003)). The studies discussed above support the notion that neural pathways involved in the reinstatement of drug seeking are likely to be different (to a certain degree) to those mediating drug reinforcement and

extinction. There are two different processes occurring during saline extinction: the first is the original association is retrieved and the second is that a novel association is formed between the previously drug-associated environment and the new pairing with saline (Bouton, 2002). The new association interferes with the original one in the process of reconsolidation (Sara, 2000). It is probable that NMDA receptor antagonists disrupt the processing of conditioned responses brought into the reactivated state during extinction. The original preference could be reinstated by a morphine prime; however in AMPA or NMDA receptor antagonist-treated animals, the original association between the effects of morphine and the drug-paired chamber has been altered so as to block reinstatement (Cornish and Kalivas, 2000, Ribeiro Do Couto et al., 2005, Ma et al., 2007, Engblom et al., 2008a).

1.3.4.2. Drug-induced reinstatement of drug seeking

Many studies have shown that drug priming injections act to renew the salience of the conditioned behaviour following abstinence (Stewart, 1983, de Wit and Stewart, 1983, Shaham and Stewart, 1996, Do Couto et al., 2003, Rogers et al., 2008). Goddard and Leri (2006) passively administered cocaine intraperitoneally to rats and paired these infusions with a compound stimulus. They were then allowed to lever press for the conditioned stimulus under extinction conditions. After extinction, a single priming injection of cocaine reinstated lever pressing for the conditioned stimulus even though rats had not directly learned to lever press for cocaine (Goddard and Leri, 2006). This finding is in agreement with the current understanding that drug priming restores the incentive value of drug related cues and triggers the reinstatement of drug seeking.

As previously discussed, most (if not all) drugs of abuse activate the dopaminergic mesocorticolimbic pathways of the brain (Luscher and Malenka, 2011). There is some evidence of D1 receptor involvement in heroin-primed reinstatement. For example, the inhibition of D1 receptors in the mPFC inhibited the reinstatement of heroin self-administration (See, 2009). The selective D2 dopamine receptor agonist bromocriptine was found to reinstate heroin seeking (Wise et al., 1990), whereas D2-like antagonists (haloperidol and raclopride) block reinstatement (Ettenberg et al., 1996, Shaham and Stewart, 1996). Others found that heroin priming causes LTP-like changes in field excitatory postsynaptic potentials in NAc core neurons after mPFC stimulation, which was mediated by the inhibition of NMDA receptors in the NAc core (Shen et al., 2011). There is extensive literature on the potential use of dopamine receptor agonists and antagonists to treat addiction, however their success is limited by their poor efficacy and the significant adverse effects associated with dopaminergic medications. Dopamine receptor agonists can be used to alleviate the negative affective state associated with withdrawal, however these drugs may also exert reinforcing and psychotomimetic effects, limiting therapeutic use (Moreira and Dalley, 2015). Dopamine receptor antagonists are theorised to restore the abnormal dopaminergic signalling observed during addiction, however anhedonia, motor impairment and neuroendocrine balance render these compounds unattractive as addiction therapies (Moreira and Dalley, 2015). Investigation into additional pathways involved in reinstatement is therefore required to identify potential targets for the more effective treatment of addiction.

1.4. Cholinergic modulation of learning and memory processes

1.4.1. Nicotinic receptors

To date, 12 genes have been identified that code for subunits of mammalian neuronal nicotinic receptors, nine α (α2-10) and three β(β2-4) (Wonnacott et al., 2005). These subunits combine in various permutations to form functional receptors, but the most widely expressed subtypes in the brain, and in the hippocampus in particular, are the homomeric α7 and heteromeric α4β2 nAChR (Albuquerque et al., 2009). These receptors have been implicated in a variety of diseases such as Alzheimer's disease, schizophrenia, depression, nicotine and alcohol addiction (Sharma and Vijayaraghavan, 2008, Feduccia et al., 2012).

1.4.2. Cholinergic circuitry

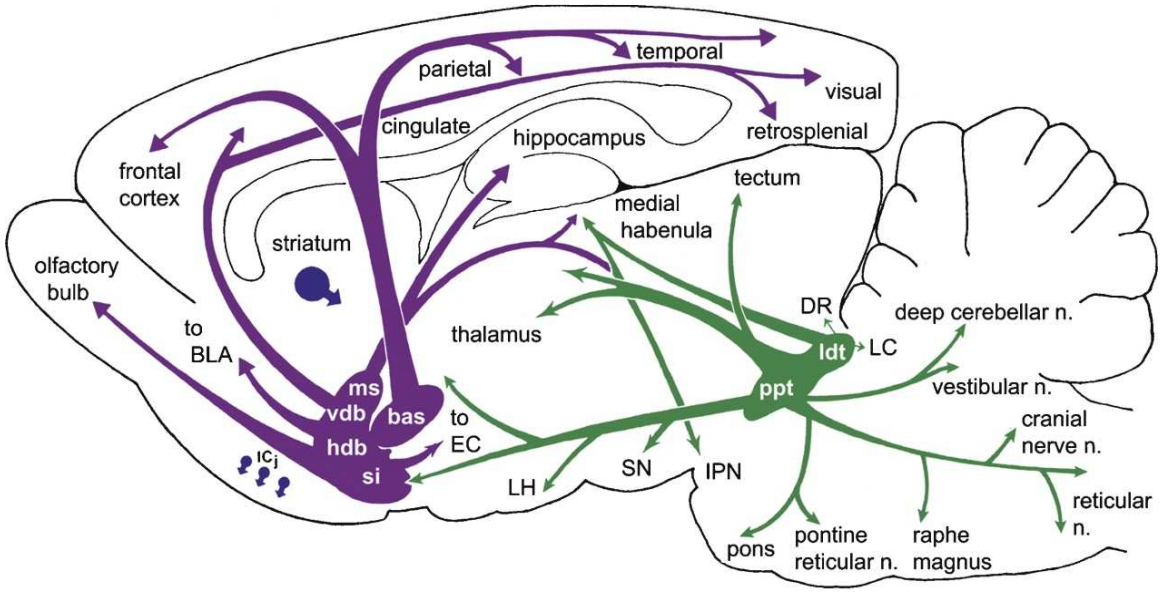


Figure 1.10: Overview of the main cholinergic pathways in the brain. Purple: cell bodies reside in the basal forebrain and project from the medial septum (ms), vertical limbs of the diagonal band of Broca (vdb), nucleus basalis of Meynert (bas) and substantia innominate (si) to the thalamus, hippocampus and throughout the cortex. The brainstem cholinergic system (green) is formed of cholinergic neurons in the laterodorsal tegmental (ldt) and pedunculopontine tegmental nuclei (ppt), which extend to the hindbrain, thalamus, lateral hypothalamus (LH) and forebrain. Cholinergic neurons in the striatum innervate local striatal neurons and the olfactory tubercle (not shown). Other abbreviations: BLA, basolateral amygdala; DR, dorsal raphe; EC, entorhinal cortex; hdb, horizontal limb of the diagonal band of Broca; lCj, islands of Calleja; IPN, interpeduncular nucleus; LC, locus coeruleus; SN, substantia nigra. (Woolf and Butcher, 2011).

Acetylcholine (ACh) acts as a primary excitatory neurotransmitter in the neuromuscular junction and the parasympathetic nervous system (Wonnacott et al., 2005). In the central nervous system, nAChRs activated by acetylcholine can be excitatory like AMPA receptors, but are more often argued to play a neuromodulatory role, by modulating the excitation state of single or groups of neurons and their responses to further stimuli (Picciotto et al., 2012).

The development of the choline acetyltransferase (ChAT) antibody in the 1980s has allowed the identification of the major cholinergic cell bodies in the brain. Using immunohistochemistry in mammalian brain slices (rat, cat and monkey); Mammalian brains have been extensively immunolabelled to identify the main brain regions encompassing large numbers of cholinergic cells, and to determine their projections to other brain regions (Woolf, 1991, Woolf and Butcher, 2011). These projections are summarised in Figure 1.10. Although ChAT-positive neurons were found irregularly dispersed throughout some regions (such as the amygdala and globus pallidus), the cholinergic cell bodies could be summarised into three main areas of the brain.

One main area is in the brainstem, more specifically the pedunculopontine tegmentum (PPT) and laterodorsal pontine tegmentum (LDT, green pathway, Figure 1.10), where cholinergic neurons innervate the thalamus and midbrain dopaminergic areas (Paul et al., 2015) and are involved in modulating REM sleep cycles, locomotor behaviour and in higher cognitive functions such as memory and gating sensory input (Garcia-Rill et al., 1987, Webster and Jones, 1988, Bosch and Schmid, 2008). Brainstem cholinergic neurons have also more recently been shown to provide direct innervation of the striatum and NAc using transgenic rats expressing fluorescent ChAT, and retrograde labelling (Dautan et al., 2014). The second major area of the brain is the basal forebrain (BF, purple pathway, Figure 1.10), where cell bodies are concentrated into four regions: the medial septum (MS), vertical limbs of the diagonal band of Broca (vDB), nucleus basalis of Meynert (NBM) and the substantia innominate (SI), where cholinergic neurons project broadly and sparsely throughout the cortex, hippocampus, thalamus and olfactory bulb (Woolf, 1991, Paul et al., 2015). These cholinergic projections

appear to be involved in memory, arousal and other cognitive processes (Wonnacott et al., 2005). Finally, there is a collection of cholinergic interneurons in the striatum (Figure 1.10) which provide extensive innervation to local striatal neurons and to the olfactory tubercle; and these are involved in a variety of behaviours including locomotor behaviours (Vrijmoed-de Vries and Cools, 1986, Kelley et al., 1989, Albuquerque et al., 2009).

Cholinergic neurons form loosely organised clusters of cells intertwined with monoaminergic neurons (Livingstone and Wonnacott, 2009). In particular, they co-exist with serotonergic neurons in the raphe nuclei, with noradrenergic neurons in the locus coeruleus (LC) and with dopaminergic neurons in the SN and VTA (Picciotto et al., 2012). Cholinergic neurons are few in numbers in the brain compared to other neuronal subtypes (glutamatergic and GABAergic, especially), but the number of cholinergic terminals in the brain is considerable as nearly all brain regions receive a cholinergic input (Woolf, 1991). Their influence on cognitive processes is therefore profound. This study will focus on the basal forebrain cholinergic projections, and more specifically, the septo-hippocampal pathway neurons projecting to the hippocampus (see Figure 1.10), due to their major roles in modulating learning and memory processes (Brayda-Bruno et al., 2013).

1.4.3. Acetylcholine receptors

There are two classes of acetylcholine receptor, the fast-transmitting ionotropic nicotinic acetylcholine receptors (nAChRs), and the slower, modulatory G protein coupled receptor muscarinic acetylcholine receptors (mAChRs). There is evidence

of mAChR involvement in cocaine, opioid and ethanol addiction (Fink-Jensen et al., 2003, Dencker et al., 2011, Berizzi et al., 2018), particularly as they have been shown to modulate synaptic plasticity (Kauer and Malenka, 2007, Citri and Malenka, 2008) however this thesis will however focus on the role of nicotinic receptors.

Nicotinic acetylcholine receptors are differentially localised within a neuron: on the soma, dendrites or synaptic terminals (Livingstone and Wonnacott, 2009). This diverse distribution of receptors can present a challenge to the functional interpretation of receptor activation; however it suggests a modulatory role of these receptors on the activity of a network rather than individual synaptic pathways. Presynaptic nAChRs induce the release of a variety of neurotransmitters when activated, such as GABA, glutamate, dopamine, 5-HT, norepinephrine and acetylcholine (Wonnacott et al., 2005, Albuquerque et al., 2009). α7-nAChRs have been shown to modulate glutamate and GABA release on presynaptic terminals, whereas heteromers govern the release of other neurotransmitters as well (Albuquerque et al., 2009). Modulation of neurotransmitter release is potentially a method of nicotinic receptor-driven control of synaptic plasticity in specific brain regions. In rat hippocampal slices, the activation of α7nAChRs mediates a burst of glutamate release events, independent of incoming action potentials (Sharma and Vijayaraghavan, 2002). This burst of glutamatergic firing results in an increase in glutamate release and CAMKII-mediated release of neurotransmitters from presynaptic terminals. α7nAChRs are calcium-permeable receptors and activation of these receptors results in the slow, long-lasting influx of calcium, resulting in the release of calcium from intracellular stores (Sharma et al., 2008). Furthermore, the burst of

transmitter release is strong enough to induce depolarisation of the postsynaptic terminal in the absence of presynaptic action potential (Sharma et al., 2008), demonstrating how α7nAChRs have a robust control of neurotransmission in the CNS.

Postsynaptic nAChRs depolarise neurons, can increase their firing rate and contribute to the expression of long-term potentiation (Albuquerque et al., 2009). A unique feature of α7nAChRs is that they are highly calcium-permeable, and as they are coupled to downstream calcium amplification mechanisms, they can trigger the release of intracellular calcium from the endoplasmic reticulum, resulting in a dramatic increase in free intracellular calcium concentrations (Dajas-Bailador and Wonnacott, 2004, Sharma and Vijayaraghavan, 2008). This key feature of α7nAChRs makes them effective modulators of downstream calcium signalling cascades.

α7nAChRs are widespread throughout the hippocampus, expressed on pyramidal neurons, granule cells and GABAergic interneurons (Figure 1.11). This review will focus on the direct α7nAChR-mediated effect on glutamatergic synapses, not the indirect effect of inhibitory interneurons activated by α7nAChRs. Acute nicotine application has been shown to increase the frequency of spontaneous miniature EPSCs in hippocampus neuronal cultures, suggesting these receptors are presynaptic (Cheng and Yakel, 2015).

1.4.4. How does acetylcholine regulate learning and memory?

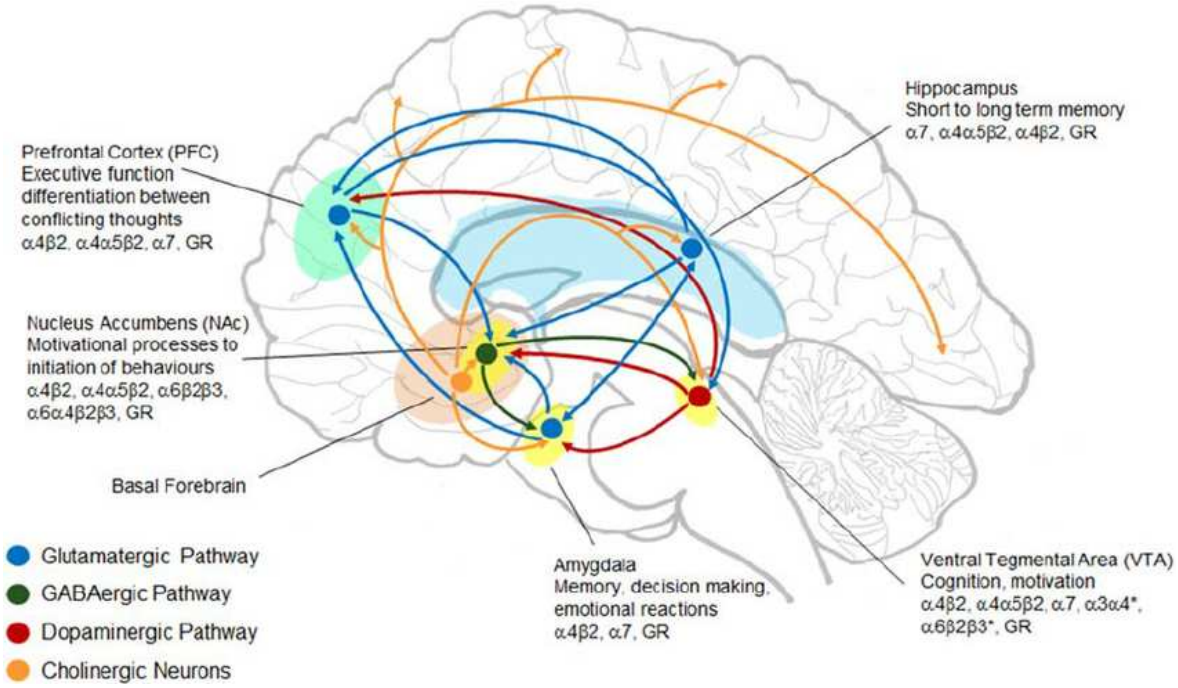


Figure 1.11: Crossover of cholinergic (orange), glutamatergic (blue), dopaminergic (red) and GABAergic (green) pathways involved in learning and memory processes and nAChR subtypes present in each brain region. Taken from Holgate and Bartlett (2015).

Basal forebrain cholinergic inputs have been demonstrated to play a major role in attention, learning, memory and consciousness. Neurotoxic lesions of the basal forebrain (BF) in rat brains by Bucci et al. (1998) induced a reduction in attention and the processing of a conditioned stimulus. Interestingly, the rats could still be conditioned to the cues when training procedures did not encourage attentional processing; which demonstrates that a level of memory processing is still intact. Other lesion studies of the BF have reported similar results in visuospatial attention (Chiba et al., 1999, Dalley et al., 2004) and spatial memory (Johnson et al., 2002, Chudasama et al., 2004).

Recently, Pitchers et al. (2017) demonstrated in a study that rats respond to motivationally salient information in different ways. They identified two types of responders: sign trackers (STs), which assert salient value to conditioned stimuli such as a lever, and have poor top-down executive control over behaviour, thought to be driven by dopamine, making them more impulsive; and goal trackers (GTs), which show more salience to the location of the reward delivery rather than the stimulus (e.g. a food cup) and display stronger top-down control over motivational behaviour, which is proposed to be controlled by cholinergic signalling (Flagel et al., 2011, Koshy Cherian et al., 2017). To test this distinction between ST and GT phenotypes, neurotoxic lesions of basal forebrain cholinergic neurons in rats were performed and their reinstatement responses to discrete cues in cocaine self-administration was measured (Pitchers et al., 2017). During reinstatement, GT animals showed significantly higher reinstatement responses to discrete cues than the ST animals. Furthermore, lesions of the basal cholinergic neurons led to the significant reduction of reinstatement in the GT group to the level seen in STs, suggesting the loss of cognitive control over motivational behaviour. The reinstatement response was the same in STs with lesioned BF cholinergic neurons and sham, suggesting no cholinergic modulation of behaviour in this group. This study shows strong evidence of basal forebrain cholinergic modulation of motivational behaviour; and in particular in the reinstatement of drug seeking behaviour; however the cholinergic receptor subtypes involved still need to be distinguished.

Lesion studies of the septo-hippocampal pathway have shown it plays a major role in spatial memory (Johnson et al., 2002). Intra-hippocampal administration of nicotine enhances working memory, whereas the α4β2 and α7nAChR-selective

nicotinic antagonists DhβE and methyllycaconitine (MLA) have an inhibitory effect (Felix and Levin, 1997, Levin et al., 2002). A microdialysis study conducted by Fadda et al. (2000) showed that acetylcholine release in the hippocampus positively correlated to spatial learning and memory. The selective lesion of septal cholinergic neurons results in a loss of theta oscillations (4-10 Hz) in the hippocampus (Apartis et al., 1998), which correlate with learning behaviours (Hernandez-Perez et al., 2016). Furthermore, the inhibition of choline uptake (to be re-synthesised into ACh) by hemicholinium-3 (HC-3) in the hippocampus of mice undergoing an inhibitory avoidance task led to long-lasting memory impairment despite choline uptake recovering 2 and 7 days post-injection (Boccia et al., 2004). This effect, and the fact that the HC-3 administration only had a deleterious effect on the behaviour immediately after memory reactivation, suggests that ACh synthesis plays a role in memory consolidation. Optogenetic activation of MS and vDB glutamatergic neurons resulted in the synchronisation of hippocampal theta oscillations in freely behaving mice, which is hypothesised to be via the modulation of local septal cholinergic pathways (Robinson et al., 2016). Moreover, in aged rats, there is significant cognitive impairment and decline which is positively correlated with a decrease in cholinergic markers in the septo-hippocampal region (Smith and Booze, 1995) and more recent lesion studies in rats show cognitive deficits, supporting these findings (Babalola et al., 2012, Brayda-Bruno et al., 2013).

These data provide evidence for a modulatory role of α7nAChRs in learning and memory processes. The next section will discuss the role of α7nAChRs in modulating reward processing.

1.4.5. α7 receptors in reward

There is extensive evidence of nicotinic receptors mediating the reinforcing effects of nicotine and being involved in nicotine dependence (Feduccia et al., 2012). The use of transgenic mice in nicotine addiction studies has revealed the differential effects of the different nicotinic receptor subtypes in regulating addictive behaviours. Mouse knockout studies of α4 or β2 subunits have revealed that while the β2 subunit is important in the acquisition of nicotine self-administration and the initial stages of nicotine dependence (reviewed by Stoker and Markou (2013)), the α4 subunit is sufficient but not essential to these behaviours (Cahir et al., 2011, Madsen et al., 2015). Indeed, the majority of smoking cessation treatments such as varenicline, cysteine and sazetidine are targeted at α4β2 nAChRs (Rahman et al., 2015).

α7 homomeric receptors appear to be involved in the later stages of nicotine addiction. α7 knockout mice are still able to acquire nicotine CPP (Walters et al., 2006b) and nicotine self-administration (Pons et al., 2008), suggesting the acquisition of drug seeking behaviour is intact. Intra-VTA infusions of nicotine however, decreased nicotine self-administration in α7 knockout mice, suggesting these receptors might be important for modulating the reinforcing properties of nicotine (Besson et al., 2012). Furthermore, in these knockout mice, dopamine levels in the VTA were significantly higher in response to nicotine administration, suggesting a regulatory role of α7nAChRs on dopamine signalling in the VTA (Besson et al., 2012).

Nicotinic receptors also modulate responses to other drugs of abuse (Rahman et al., 2015). For example, nicotinic receptors have been investigated as targets for treating alcohol addiction, as they have been shown to modulate the rewarding

effects of ethanol (Lě et al., 2006), and the non-selective nAChR antagonist mecamylamine has been shown to reduce ethanol self-administration and preference in rats (Ericson et al., 1998) and in humans (Young et al., 2006). In studies using cocaine, mecamylamine was shown to inhibit cocaine self-administration (Levin et al., 2000) and decrease the acquisition of cocaine CPP (Zachariou et al., 2001), while DhβE reduced behavioural sensitisation to cocaine in rats (Champtiaux et al., 2006), involving nicotinic receptors in cocaine addiction and seeking behaviour. Chronic administration of low doses (0.03 and 0.1 mg/kg, p.o.) of varenicline (α7 agonist and α4β2*, α3β4 and α6β2partial agonist) had no effect on cocaine self-administration in primates, whereas higher doses (0.3 and 0.56 mg/kg, p.o.) potentiated self-administration (Gould et al., 2011). Mecamylamine however had no effect on cocaine self-administration in monkeys (by either p.o., i.m. or i.v. administration), however the doses used were lower than the study performed in rats (Levin et al., 2000) and the administration of mecamylamine was chronic in the primate study rather than acute in the rodent study.

1.4.6. α7nAChRs in reinstatement models

Secci et al. (2017) used a kynurenine 3-monooxygenase (KMO) inhibitor, Ro 61-8048 to elevate kynurenic acid levels in the brain, as kynurenic acid is hypothesised to act as a negative allosteric modulator of α7nAChRs. They used Ro 61-8048 to investigate its role in the reinforcement and reinstatement of nicotine and cocaine self-administration in rats and squirrel monkeys. They found that Ro 61-8048 dose-dependently inhibited nicotine self-administration in rats and monkeys (but not cocaine in monkeys), and the selective α7nAChR positive

allosteric modulator (PAM) PNU120596 restored this response in monkeys but not in rats, suggesting additional mechanisms to the downregulation of α7nAChRs may be exerting this effect on the acquisition of self-administration (Secci et al., 2017). During reinstatement, Ro 61-8048 also significantly inhibited both drug and cue-primed reinstatement of nicotine IVSA (in rats and monkeys) and also cocaine self-administration (only in monkeys), and the α7nAChR PAM PNU120596 rescued the effect of Ro 61-8048. This selective recovery of reinstatement by the α7nAChR PAM suggests more a selective modulation of reinstatement by α7nAChRs. A previous study also demonstrated a similar effect of kynurenic acid on cannabinoid self-administration and the reinstatement of cannabinoid seeking (Justinova et al., 2013). These findings potentially support a selective role of α7nAChRs in reinstatement, though there is limited research using kynurenic acid and as kynurenic acid also antagonises glutamate receptors in the brain (Albuquerque and Schwarcz, 2013), further work is needed to elucidate these findings further.

Studies conducted by Liu et al showed that the nonselective nAChR antagonist mecamylamine and α4β2 nAChR antagonist DhβE significantly inhibited lever pressing during the acquisition of nicotine self-administration (Liu et al., 2007, Liu, 2014). The α7nAChR-selective antagonist MLA, however, had no effect at this stage (Liu et al., 2007). Conversely, DhβE had no effect on the cue-primed reinstatement of nicotine IVSA, whereas MLA significantly and dose-dependently (2.5 and 10 mg/kg, i.p.) decreased the reinstatement of nicotine seeking (Liu, 2014). These findings support differential roles for nAChR subtypes in nicotine reinforcement and cue-induced reinstatement of nicotine IVSA, and data from mouse knockout studies with respect to nicotine addiction (Stoker and Markou,

2013). This group has also identified an interaction of opioidergic and cholinergic pathways in the reinstatement of nicotine IVSA.

These key findings have identified an intriguing role of α7nAChRs, which appear to selectively modulate the reinstatement of drug (nicotine and cocaine) seeking. This has yet to be shown in the reinstatement of seeking with opioid drugs and warrants further investigation. This study will focus on the role of α7nAChRs in the modulation of opioid seeking behaviour due to previous literature cited below.

1.5. Work leading up to this thesis project

1.5.1. MLA inhibits morphine-primed reinstatement of CPP

We and others have shown that MLA, a potent and selective α7 nicotinic acetylcholine receptor antagonist, blocks morphine-primed reinstatement of CPP (as a correlate for relapse in response to contextual cues in humans) in mice and rats (Feng et al., 2011, Wright et al., 2018). Feng et al. (2011) found that MLA (4mg/kg, s.c.) pre-treatment 20 minutes prior to morphine-primed reinstatement significantly reduced the time spent in the drug-paired side in BALB/c mice. Interestingly, this inhibition of reinstatement lasted for three days after reinstatement, as subsequent morphine-primed reinstatement sessions were also inhibited in the absence of further MLA pre-treatment. Further work by Wright et al. (2018) extended the inhibition of morphine-primed reinstatement by MLA to C57BL/6J mice and Wistar rats. Furthermore, this study showed that MLA had no effect on either the acquisition, expression, reconsolidation or extinction stages of CPP (Wright et al., 2018). These findings are compelling and suggest a possible

selective role of α7nAChRs in the reinstatement of CPP, which warrants further research.

The overarching aim of this thesis is to gain a deeper understanding of the role of α7nAChRs in opioid dependence and relapse to inform potential therapeutic strategies. Morphine is a drug which is less commonly abused by addicts than other opioids, mostly due to its low potency and slow pharmacokinetics (Gottås et al., 2013). Heroin is more potent and reinforcing, with a rapid onset of action (Gottås et al., 2013) leading to it being widely abused, and is therefore a more relevant opioid in which to study the effects of MLA in behavioural paradigms of drug-seeking. The aim of this study is to extend findings on the role of α7nAChRs in the reinstatement of drug-seeking in behavioural paradigms used as human correlates of drug-seeking and relapse (Feng et al., 2011, Justinova et al., 2013, Liu, 2014, Secci et al., 2017, Wright et al., 2018). The CPP paradigm is a model of passive drug seeking triggered by contextual cues, whereas the IVSA paradigm is a more active model of drug seeking with volitional control of drug intake. Both of these paradigms may reveal intriguing aspects of the modulation of the reinstatement of drug seeking by α7nAChRs and should therefore both be studied.

1.5.2. α7nAChRs modulate AMPA receptor expression in the ventral hippocampus during morphine-primed reinstatement of CPP

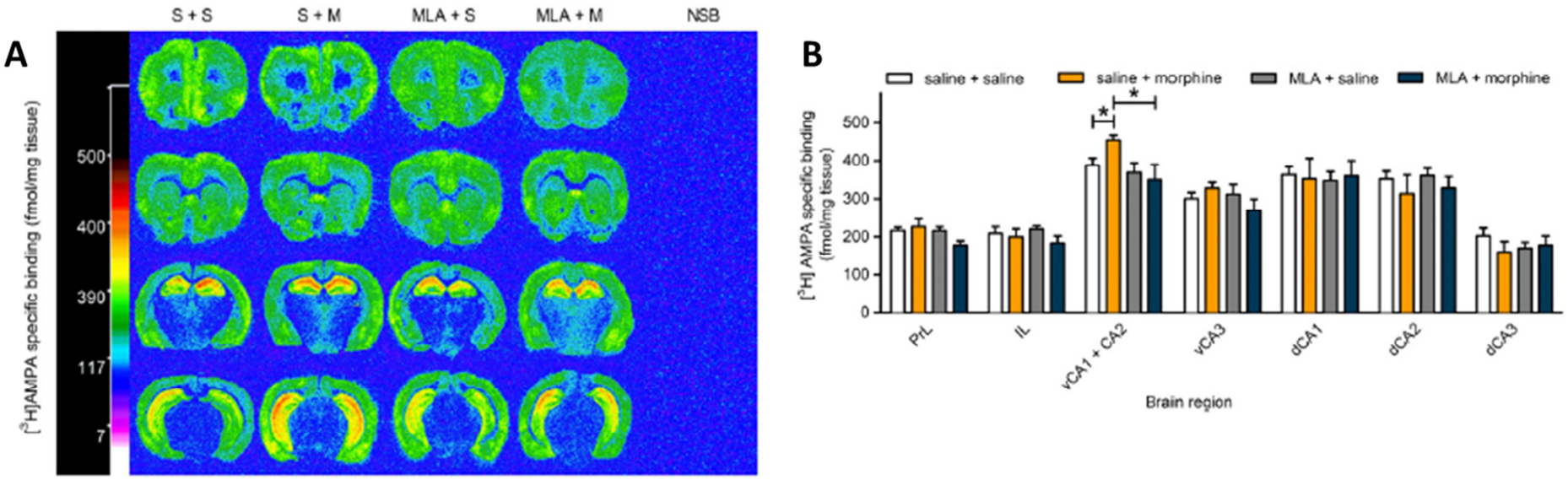


Figure 1.12: **A**: Representative [^{3}H]-AMPA binding density autoradiogram of binding following either MLA pre-treatment or morphine-primed reinstatement. Panels show coronal sections of the mPFC (top, bregma 1.94mm), striatum (second row, bregma 1.42mm), dorsal hippocampus (third row, bregma -1.22mm) and ventral hippocampus (bottom, bregma -3.08mm). **B**: Quantitative AMPA receptor binding (fmol/mg tissue equivalent) shown for prelimbic cortex (PrL), infralimbic cortex (IL), ventral hippocampus (vCA1+CA2, vCA3), dorsal hippocampus (dCA1, dCA2, dCA3). Adapted from Wright et al. (2018)

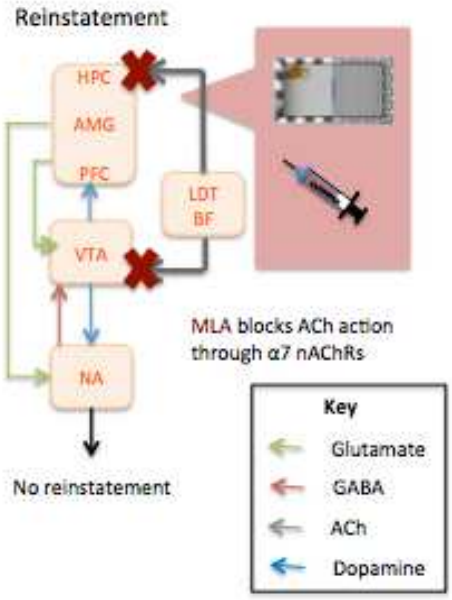


Figure 1.13: Proposed model for the role of α7nAChR in modulating glutamatergic signalling in drug-primed reinstatement of CPP. During reinstatement, exposure to a priming dose of drug causes increased glutamatergic and dopaminergic signalling leading to restored LTP and the restoration of the drug-associated memory (following extinction), consequently triggering reinstatement. MLA blocks the action of ACh in the ventral hippocampus, which results in the inhibition of excitatory and inhibitory signalling required for LTP and the expression of the drug-seeking behaviour (Wright, 2016).

Recent data from our lab have shown a significant increase in [^{3}H]-AMPA binding (Figure 1.12A) – but not [^{3}H]NMDA binding – only in the ventral hippocampus after morphine-primed reinstatement of CPP, compared to the PFC (shown in Figure 1.12B), other cortical regions, the dorsal hippocampus, CPu, NAc, amygdala and VTA (supplemental data shown in Wright (2016)); which is suggestive of an increase in AMPA expression. This increase in AMPA expression is postulated to be associated with the restoration of LTP and the drug-related memory triggered by the priming dose of morphine (see Figure 1.13). Interestingly, systemic treatment with MLA prior to morphine-induced reinstatement blocked this increase

in [^{3}H]-AMPA binding (Figure 1.12A and B), suggesting α7nAChR modulation of the restoration of LTP in reinstatement, particularly in the ventral hippocampus. Autoradiography however does not distinguish cell surface from intracellular receptor expression, therefore it cannot be determined from these results than the increase in [^{3}H]-AMPA binding correlates with an increase synaptic AMPA receptors, which underlies LTP. An additional aim of this thesis was therefore to address the limitations of autoradiography, which will be discussed further below.

1.5.3. Aims of the Thesis

There is a growing body of evidence that implicates α7nAChRs in modulating drug-associated behaviour (Rahman et al., 2015). The effects of methyllycaconitine (MLA) have been investigated in morphine CPP in mice and rats. The aim of this thesis is to extend this to a more relevant and reinforcing opioid, heroin.

The aims of this thesis were to:

1) Validate a heroin CPP model

Heroin has rarely been used in conditioned place preference experiments. Of the few research papers studying heroin CPP (see Bardo et al. (1995) and Tzschentke (2007) for comprehensive reviews), only 2 to my knowledge studied heroin-primed reinstatement of heroin CPP (Leri and Rizos, 2005, van der Kam et al., 2009). This is partly due to the fact that in some countries, heroin is a restricted substance, whereas morphine is more widely available. In the first Results chapter (Chapter 2) of this thesis, validation of a heroin conditioned place preference model in rats is described.

2) Study the role of α7nAChRs in heroin-primed reinstatement of CPP

The rat heroin CPP model was then utilised to investigate the role of α7nAChRs in heroin-primed reinstatement of CPP to extend results found by Feng et al. (2011) and Wright et al. (2018) in mouse morphine CPP. Results in Chapter 2 describe the effect of MLA on heroin-primed reinstatement of CPP. Based on previous literature and previous work in this lab, it was hypothesised that MLA would inhibit the heroin-primed reinstatement of CPP in rats.

3) Optimise and validate methods of synaptic AMPA receptor quantification following reinstatement

The location of α7nAChRs puts them in prime location to modulate synaptic plasticity implicated in motivational learning (Albuquerque et al., 2009), and previous findings by Wright et al. (2018) revealed a modulatory effect of α7nAChRs on total AMPA receptor expression in the ventral hippocampus in morphine-primed reinstatement of CPP. To investigate this role further, different methods of AMPA receptor isolation and quantification at the synapse were optimised and validated (Appendix A and B) to enable the quantification of AMPA receptors in the synapse following heroin-primed reinstatement of CPP in rats (Chapter 2). Based on previous findings in this lab (Wright et al., 2018), it was hypothesised that heroin-primed reinstatement would be correlated with an increase in synaptic GluA1 and GluA1 phosphoSer845.

4) Study the role of α7nAChRs on heroin self-administration behaviours

In Chapter 3, the effects of MLA were examined in this operant model of addiction, to complement the CPP study. The role of α7nAChRs on heroin IVSA was examined by studying the effect of MLA on the acquisition of lever pressing, the heroin- and cue-primed reinstatement of IVSA, the relative reinforcement of heroin (breakpoints of progressive ratio) and on the re-acquisition of heroin IVSA. It was hypothesised that as MLA inhibited the heroin-primed reinstatement of CPP but had no effect on the acquisition of heroin CPP (Chapter 2), it would inhibit the heroin- and cue-primed reinstatement of IVSA but have no effect on lever pressing behaviour or on the motivational value of heroin.

CHAPTER 2 THE ROLE OF α7NACHRS DURING HEROIN-PRIMED REINSTATEMENT OF CONDITIONED PLACE PREFERENCE

2.1. Introduction

2.1.1. Conditioned place preference

A current major failure of addiction treatment is the lack of prevention of cravings which lead to relapse. Repeated drug consumption with the same contextual cues (locations, people, paraphernalia etc.) results in drug-paired associations which become cues for cravings (see Chapter 1, section 1.3.). In vivo behavioural paradigms are used to model various aspects of these drug-paired associations and how they can trigger relapse-like behaviour. This chapter describes the use of conditioned place preference (CPP) as a correlate of drug-associated memories and the role of α7nAChR in the reinstatement of this model.

2.1.2. CPP as a correlate of drug-associated memory

As described in Chapter 1 (Section 1.4.1) the CPP paradigm is a form of Pavlovian conditioning used to study motivational memory associated with a rewarding or aversive stimulus (for review, see Cunningham et al. (2006)). CPP has face validity for addiction processes in humans, as addicts report strong associations with environments in which they abuse drugs, which can trigger cravings (Bardo and Bevins, 2000). In fact, a human model of CPP has been explored (Childs and de Wit, 2009, 2013), where humans demonstrate a preference for a room in which they receive d-amphetamine compared to another room in which they receive placebo. The fact that CPP can be produced in humans reinforces the face validity of the rodent model, however it is important to remember that CPP is a correlate of a specific type of addiction-related behaviour which contributes to relapse, but it does not model addiction and relapse as a whole, especially as in CPP, the

behaviour is involuntary. However, there are many instances of drugs which have been shown to interfere with CPP in preclinical studies being FDA-approved addiction treatments, demonstrating the predictive validity of CPP (for review, see Tzschentke (2007)).

CPP is limited by some factors. One concern is that some animals show an initial preference for one compartment during habituation (Napier et al., 2013); however using a balanced unbiased protocol can reduce the influence of pre-existing preference or aversion on the interpretation of results. The CPP procedure has also been argued to be relatively insensitive to drug dose, which potentially reduces its usefulness when measuring the value of a drug during conditioning (Napier et al., 2013). However the response magnitude is less important when measuring drugs that interfere with the expression of CPP once the behaviour has been acquired, and also when using priming stimuli to induce reinstatement after extinction. This distinction is clinically relevant as a drug that interferes with the conditioned behaviour in a drug-free state is more likely to reduce context-induced craving or relapse in an abstinent human. The focus of this thesis is to manipulate the drug-associated cues which trigger the reinstatement of drug-seeking behaviour therefore the magnitude of CPP is less important here.

2.1.3. Nicotinic receptor role in opioid addiction

There is evidence of interactions between opioids and nicotinic receptors. A study showed that methadone has non-competitive antagonist actions at α4β2 nAChRs and competitive antagonist actions at α7nAChRs in [^{3}H]-epibatidine radioligand binding assays (Talka et al., 2015). Furthermore, methyllycaconitine (MLA), the

selective α7nAChR antagonist, blocked methadone-induced elevations of $[Ca^{2+}]_i$ in calcium fluorescence assays. These results suggest a potential effect of methadone on nAChRs, however methadone has been shown to block various ion channels at medium and high concentrations (Matsui and Williams, 2010, Stoetzer et al., 2015), suggesting this effect may not be generalised to all opioids.

In a model of withdrawal-induced conditioned place aversion (CPA), Ise et al. (2000) found that morphine pre-treatment could prevent mecamylamine-precipitated nicotine withdrawal CPA in rats chronically treated with nicotine. In addition, they showed that the non-selective opioid antagonist naloxone could also dose-dependently precipitate nicotine withdrawal CPA in these rats chronically treated with nicotine (Ise et al., 2000). Biala and Weglinska (2004) found that morphine, nicotine and MK-801 (an NMDA receptor antagonist), but not cocaine or amphetamine, enhanced locomotion in nicotine-experienced mice, which was blocked by L-type voltage-gated calcium channel blockers (Biala and Weglinska, 2004). Furthermore, Suh et al. (1996) found that nicotine enhanced the anti-nociceptive effects of morphine in tail-flick experiments in mice. In a passive avoidance task for the assessment of memory impairment, another study found that nicotine improved morphine-induced memory impairment (Ahmadi et al., 2007). These results suggest an interaction of the nicotinic and opioidergic systems in addiction.

There is also extensive evidence of cross-reinstatement; the phenomenon of drugs other than those previously received which reinstate drug-seeking behaviour. Biala and Budzynska have repeatedly shown that morphine induced the reinstatement of nicotine CPP to the same extent as nicotine (Biala and Budzynska, 2006, Biala et al., 2009, Biala and Budzynska, 2010). They also

showed ethanol could reinstate nicotine CPP in mice (Biala and Budzynska, 2010). To date, there is no evidence that nicotine priming reinstates morphine CPP (Feng et al., 2011), however Zarrindast et al. (2003) demonstrated that repeated doses of either morphine or nicotine to induce tolerance reduced the place conditioning effect of both morphine and nicotine in mice, suggesting some cross-tolerance between the effects of the two drugs and an interaction between opioid and nicotinic modulation of reward-seeking behaviour. This cross-tolerance was also observed by Vihavainen et al. (2008), who hypothesised this effect was not mediated by μ opioid receptors (MORs) due to no change in the number or affinity of MORs following nicotine sensitisation by [^{3}H]-DAMGO autoradiography in multiple brain regions.

The mesocorticolimbic dopamine system is considered by some the major substrate of reward and reinforcement associated with drugs of abuse and natural rewards (Chapter 1, section 1.2.1, (Hyman et al., 2006, Stewart, 2008, Koob, 2013)) and nicotinic receptors modulate dopaminergic signalling in these reward systems (Balfour, 2004, Hyman et al., 2006, Vihavainen et al., 2008). However other pathways (notably glutamatergic, see Section 1.2.3.) play increasingly important roles in the later stages of addiction, as there is little concrete evidence of dopaminergic signalling during the reinstatement of CPP (Section 1.4.4.).

2.1.4. Evidence of glutamatergic signalling in the hippocampus in CPP

As previously discussed in Chapter 1 (section 1.4.4.), there is extensive evidence of glutamatergic pathways modulating reward processes throughout the brain.

NMDA receptor involvement has been extensively studied in the modulation of morphine CPP. NMDA receptor antagonists have been shown to block the acquisition (Tzschentke and Schmidt, 1995, Zarrindast et al., 2007, Rezayof et al., 2007, Kao et al., 2011), expression (Tzschentke and Schmidt, 1997, Rezayof et al., 2007, Portugal et al., 2014) and reinstatement (Ribeiro Do Couto et al., 2005, Popik et al., 2006, Ma et al., 2007, Portugal et al., 2014) of morphine CPP, involving brain regions such as the dorsal hippocampus (in acquisition, extinction and reinstatement), central amygdala (acquisition and expression), and NAc (acquisition and reinstatement). These data strongly indicate an important role of glutamatergic pathways in modulating opioid reward.

Studies have shown that synaptic remodelling in the hippocampus is essential for the processing of cue-associated memories in the context of drug-related behaviours. Portugal et al. (2014) have shown that the acquisition of morphine CPP is associated with impaired LTP but increased basal synaptic transmission in field recordings in mouse hippocampus slices. Another study corroborating this finding reported a decrease in the number of dendritic spines in the CA1 region of the hippocampus following the acquisition of morphine CPP in mice (Fakira et al., 2016); however, unpaired morphine administration also resulted in a reduction in dendritic spines, suggesting non-specific effects of morphine. The acquisition of morphine CPP was however shown to correlate with a significant increase in fos expression in the dentate gyrus of the septotemporal axis of the hippocampus (Rivera et al., 2015). Extinction is also associated with the disruption of LTP in the hippocampus, but morphine-primed reinstatement results in the robust enhancement of LTP in hippocampal slices, which is blocked by the local antagonism of GluN2B NMDA receptor subunits (Portugal et al., 2014), suggesting

this enhancement of LTP is NMDA receptor dependent. Furthermore, the inhibition of dorsal hippocampus activity by the administration of a $GABA_A$ agonist muscimol leads to the impairment of the acquisition, extinction and expression of cocaine CPP (Hitchcock and Lattal, 2018). These findings strongly support an important role of the hippocampus in all stages of CPP, though the majority of studies are conducted in the dorsal hippocampus.

This thesis focuses on the role of synaptic plasticity in the ventral hippocampus in the reinstatement of CPP as previously in our lab, it was demonstrated that intracerebral infusions of the selective α7nAChR antagonist MLA into the ventral hippocampus only, resulted in the inhibition of morphine-primed CPP in rats (Wright et al., 2018). Furthermore, morphine-primed reinstatement of CPP was associated with a significant increase in [^{3}H]-AMPA binding in the ventral hippocampus only, which was blocked by MLA (Wright et al., 2018).

3.1.1. Evidence of α7nAChR in the reinstatement of CPP

As discussed in Chapter 1 (sections 1.4.5 and 1.5), there are some reports suggesting a role of α7nAChR in CPP. A study found that the non-selective nAChR antagonist mecamylamine and the α4β2nAChR antagonist DhβE both inhibited the acquisition of nicotine CPP in mice, whereas the α7nAChR-selective MLA had no effect (Walters et al., 2006b). Furthermore, α7nAChR knockout mice were still able to acquire nicotine CPP, compared to β2 knockout mice, which were not (Walters et al., 2006b). Mice with α7nAChR knocked out from the NAc were unable to acquire nicotine CPP, but cocaine CPP was intact, suggesting a there

could be a role of α7nAChRs in the reinforcement of nicotine that is not widespread across all drugs of abuse (Harenza et al., 2014).

Interestingly, recent studies have shown that MLA pre-treatment significantly attenuates morphine-primed reinstatement of CPP in mice and rats (Feng et al., 2011, Wright et al., 2018). Wright et al. (2018) further demonstrated that MLA had no effect on the acquisition, expression, reconsolidation and extinction of morphine-primed reinstatement, which suggests an intriguing selective role of α7nAChRs in the reinstatement of CPP. In addition, morphine-primed reinstatement was associated with a significant increase in total [^{3}H]-AMPA binding in the ventral hippocampus, which was blocked by MLA (Figure 1.12). These findings are compelling and form the basis of this thesis.

2.2. Aims of this chapter

The main aim of this chapter was to investigate the role of α7nAChRs on the reinstatement of heroin-primed CPP in rats, extending previous findings with morphine (Feng et al., 2011, Wright et al., 2018). After establishing heroin CPP in rats, we hypothesised that:

1) MLA would have no effect on the acquisition of heroin CPP

This was investigated by MLA pre-treatment during conditioning of heroin CPP.

2) MLA would inhibit heroin-primed reinstatement of CPP

This was investigated by MLA pre-treatment 20 minutes prior to heroin-primed reinstatement of CPP.

The secondary aim of this chapter was to optimise and validate a method for quantifying synaptic AMPA receptors in brain slices from rats that underwent heroin-primed reinstatement; at a more sensitive subunit-specific level. We hypothesised that:

1) Heroin-primed reinstatement would be associated with an increase in GluA1 and phosphorylated GluA1 (Ser845) at synaptic regions in the ventral hippocampus
2) MLA pre-treatment would inhibit the reinstatement-induced increase in synaptic AMPA receptor expression

This was investigated by the fixation of rat brains that underwent heroin-primed reinstatement of CPP, followed by immunolabelling of ventral hippocampus slices and visualisation by Li-Cor near-infrared scanning technology and confocal microscopy.

2.3. Materials and Methods

2.3.1. Statistical Analysis

All CPP data are presented as mean ± standard error of the mean (SEM). Data were collected via a camera and analysed through a PC equipped with an auto-monitoring system (Ethovision XT version 8.0). The conditioned place preference scores are presented as time spent in the drug-paired side-450 seconds (half of the post-test time) to give a preference score that represents an increase in time spent in the drug-paired side. Exclusions were made post-extinction if animals spent over 65% (10 minutes) of the total post-test test time (based on Cordery et

al. (2014)). Statistical outliers in each stage of conditioned place preference were determined using Grubbs' test for outliers. During reinstatement, the last 15 minutes of the reinstatement test was used for comparison with the post tests, as it has previously been shown by time course analysis of the reinstatement of morphine CPP that rats show more preference during the second half of the 30 minute test (Mueller et al., 2002). All behavioural analysis was done in GraphPad Prism 5 by one- or two-way ANOVA with Bonferroni post-hoc analysis, where appropriate.

Immunohistochemistry data were presented as means ± SEM. Two-way ANOVA analysis was used to determine an effect of treatment or differences between regions of the ventral hippocampus on the expression levels of total GluA1 and phosphorylated GluA1 at serine845.

2.3.2. Drugs

For CPP experiments, all drugs were dissolved in sterile sodium chloride solution (0.9% w/v, Hameln pharmaceuticals, Gloucester, UK) and filter sterilised using 0.45 µm syringe filters (Millipore). Injections were administered subcutaneously (s.c.) and (heroin, MLA and saline) were given using a dose volume of 1mL/kg). The injection site (left or right side of the body) was alternated to allow the skin to recover before the next injection. Heroin hydrochloride (McFarlan Smith, UK) was stored in a locked cabinet while not in use. Heroin injections for conditioning and reinstatement (1mg/kg) were made up at 1 mg/mL and stored in 1 mL aliquots in sterile tubes at -20℃ for up to one month. Methyll ycaconitine citrate (MLA, Abcam) was made up into 4mg/mL 1mL aliquots; which were then stored at -20℃

for a maximum of one month. Drugs were thawed at room temperature at least 30 minutes before dosing. Aliquots were used once and were not returned to -20℃ to prevent freeze-thawing of drugs. Heroin was used at a dose of 1mg/kg for both the conditioning and reinstatement phases as previous studies showed this dose was effective in producing CPP (Leri and Rizos, 2005, Tzschentke et al., 2006, Galaj et al., 2015, Galaj et al., 2016) and causing reinstatement (Leri and Rizos, 2005)

2.3.3. Animals

All experiments were performed in accordance with Home Office project licence held under 'ASPA' 1986 and approved by a local ethical review panel. Male Wistar rats (5-9 weeks) were bred from the University of Bath breeding colony. For CPP experiments, all animals were housed in fours in a behavioural holding room with controlled temperature (24±2°C), humidity (50-60%), and a 12:12h light-dark cycle (lights on 0700-1900). Food (standard rat chow) and water was available *ad libitum*. All animals were allowed to habituate to laboratory conditions for one week before experiments began during which they were handled daily in the experimental room. For the reinstatement of heroin CPP experiment, 48 rats were used before exclusions. For the effect of MLA on the acquisition of heroin CPP, 24 rats were used (12 per treatment group). Animals were tested Monday-Friday and cages were cleaned weekly on Fridays after testing to allow a two day recovery over the weekend before the next stage of the behavioural protocol.

2.3.4. CPP apparatus

The apparatus (MED Associates, UK) consisted of a two-compartment box (40x40 cm each), one with vertical and one with horizontal black and white stripes, separated by removable guillotine doors and a central neutral zone measuring (10x20 cm). The floors of each chamber were also different, one with 2 cm round holes or 1x1 cm square holes respectively (as shown in Figure 2.1).

Figure 2.1: Rat CPP apparatus **Left panel**: rat CPP apparatus comprising of three compartments: one with circular holes in the floor and horizontal black and white stripes on the wall, one neutral middle compartment with a smooth, dark floor and one with square holes in the floor and vertical black and white stripes on the wall. **Right panel**: Layout of CPP boxes in the experimental room. CPP boxes are placed side by side, with alternate sides of the apparatus facing the wall in case of preference to one side of the room. Boxes were covered with Perspex lids to minimise noise and prevent rats from escaping. The boxes were places on rails with plastic sheets underneath to facilitate cleaning. Lights were dimmed by placing cardboard in front of the lamps, as seen in the right corner of the room. Cameras were mounted to the ceiling to record behaviour in 2 boxes per camera.

2.3.5. CPP Procedures

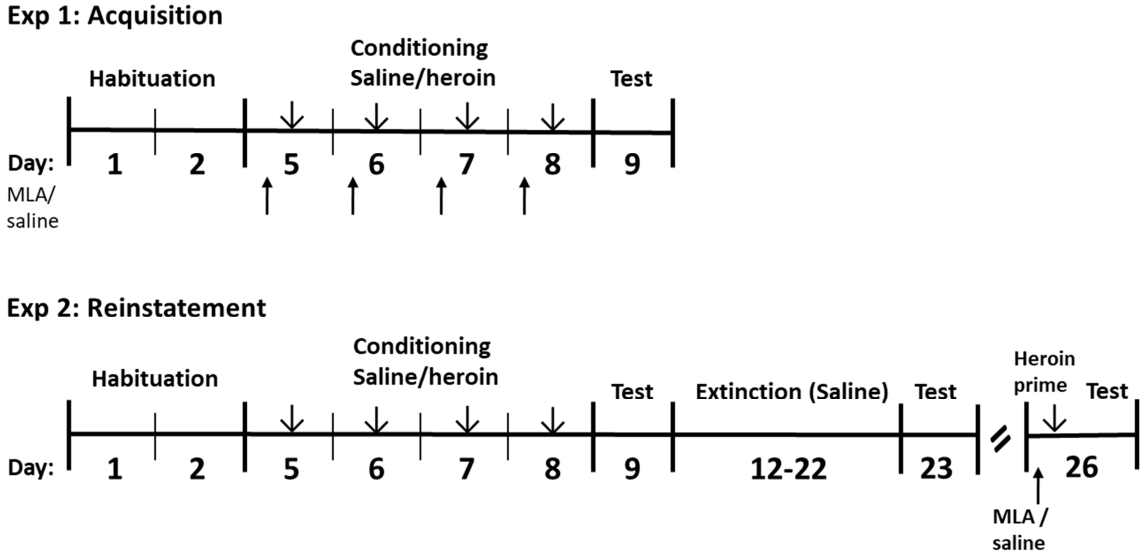


Figure 2.2: Diagram of the heroin CPP protocols for experiment 1 (top): the effect of MLA on the acquisition of heroin CPP, and experiment 2 (bottom): the effect of MLA on the heroin-primed reinstatement of CPP. Downward arrows ↓ represent saline or heroin injections during conditioning. Upward arrows ↑ represent MLA (or saline) injections 20 minutes prior to heroin.

The CPP procedure consisted of six different trials: the Habituation trial (1x15 minute session/day for 2 days); Conditioning (1x40 minute trial/day for 4 days), Post-conditioning test (1x15 minute trial), Extinction (1x30 minute trial/day for 4 days for mice and for 9 days for rats), Post-extinction test (1x15 minute trial) and Reinstatement (1x30 minute trial). The whole protocol and the steps used for each experiment are shown in Figure 2.2. Data was collected via a camera and analysed through a PC equipped with an auto-monitoring system (Ethovision XT version 8.0).

2.3.5.1. Habituation

On the first day of the study (Figure 2.2), each animal was placed in the CPP chamber and allowed to move freely between the two compartments for 15 minutes, whilst the cameras tracked their movements. The software tracked the time spent in either compartment (in seconds), and the total distance moved (in centimetres), which determined any initial preference or aversion to one particular compartment. This test was then repeated the next day and the means of the data obtained on both days allowed the assignment of treatment groups in a pseudo-randomised trial for a counterbalanced design, where half the animals were conditioned to the horizontal-striped compartment, and half to the vertical-striped compartment; but also, where preference for a particular compartment was balanced by conditioning half the animals to their preferred side and half to their non-preferred side (if preference was shown during the habituation tests).

2.3.5.2. Conditioning

On day 5 (see Figure 2.2), rats received either saline (1mL/kg, s.c.) or heroin (1 mg/kg, s.c.) and were restricted to their corresponding compartment for 40 minutes. On the following day, those that received heroin received saline and were restricted to the opposite side of the CPP apparatus. This was repeated over four days so rats all received two injections of heroin and two injections of saline during the conditioning sessions. After each session, the rats were returned to their home cages until the following day.

2.3.5.3. MLA pre-treatment

The effects of MLA on the acquisition of heroin CPP in rats was tested in a separate cohort of 24 male Wistar rats (6-7 weeks old). After habituation, animals were pseudo-randomly allocated to one of two treatment groups. Group 1 received MLA (4 mg/kg, s.c.) 20 minutes prior to heroin conditioning (1 mg/kg, s.c.) and group 2 received saline (1 ml/kg, s.c.) 20 minutes prior to heroin conditioning (1 mg/kg, s.c.) and were then placed in the conditioned side for 40 minutes (as discussed in the previous section). Rats were also pre-treated before saline administration in the unpaired side. This was repeated over 4 days as shown in Figure 2.2. All pre-treatments and conditioning doses were administered daily in the experimental room and in between the pre-treatment and treatment injections animals were returned to their home cages.

2.3.5.4. Post-conditioning test

24 hours after the last conditioning day, the guillotine doors were removed and animals were allowed to roam freely in the CPP apparatus for 15 minutes. The time spent in each chamber and the total distance moved was recorded and analysed using the Ethovision software. A preliminary paired t test compared conditioning scores to habituation to determine the success of conditioning before proceeding to the following step.

2.3.5.5. Extinction

Two days after the post conditioning test, rats received daily injections of saline (1mL/kg, s.c.) for 9 days (days 12-22, excluding the weekend, see Figure 2.2) and were restricted to either side of the CPP apparatus on alternating days.

2.3.5.6. Post-extinction test

24 hours after the last extinction session, the guillotine doors were removed and animals were allowed to roam freely in the CPP apparatus for 15 minutes and were tracked using the Ethovision software. Only animals meeting the criterion of <65% of the total post-test time (Cordery et al., 2014) were used in the reinstatement phase.

2.3.5.7. Reinstatement

On reinstatement day (day 26, see Figure 2.2), rats were randomly assigned to one of four treatment groups. **Figure 2.3** shows the injection protocol for the treatment groups described below. Groups received either saline or MLA 20 minutes prior to heroin or saline priming. Treatment groups were: the saline control group (S+S), which received saline (1 mL/kg, s.c.) followed by a priming dose of saline (1 ml/kg, s.c.); the heroin reinstatement group (S+H), which received saline (1 ml/kg, s.c.) before a priming dose of heroin (1mg/kg, s.c.); the MLA control group (MLA+S), which received MLA (4mg/kg, s.c.) followed by a saline priming dose (1 mL/kg, s.c.) and the MLA reinstatement group (MLA+H), which received MLA (4mg/kg, s.c.) before a priming dose of heroin (1 mg/kg, s.c.).

Rats were placed free-roaming in the CPP apparatus immediately after their priming dose and were recorded for 30 minutes for a single reinstatement session.

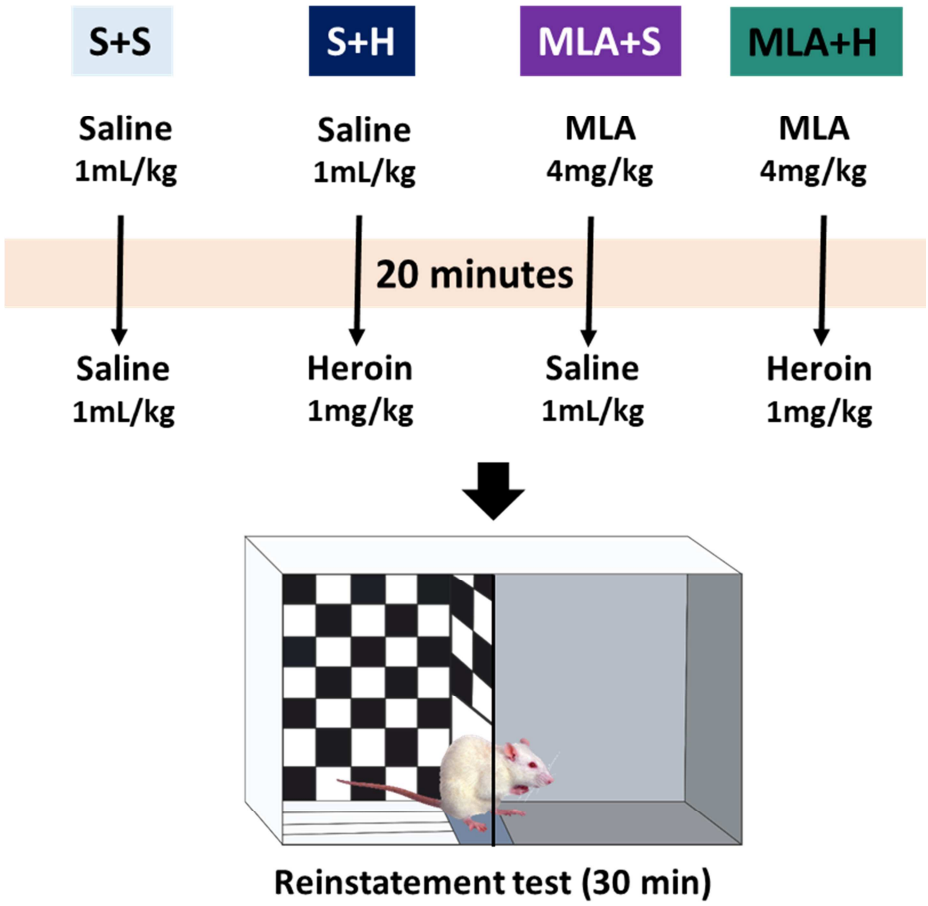


Figure 2.3: Injection protocol investigating the effect of MLA pre-treatment on the heroin-primed reinstatement of CPP. Treatment groups were: S+S: Saline control; S+H: heroin-primed reinstatement; MLA+S: MLA control; MLA+H: MLA reinstatement. All drugs were administered subcutaneously at a dosing volume of 1mL/kg.

2.3.6. Immunohistochemistry

2.3.6.1. Perfusion fixation

Immediately following reinstatement, rats were perfused with paraformaldehyde (PFA) under terminal anaesthesia. Animals were anaesthetised by the inhalation anaesthetic isoflurane (Abbott, 2-3 cc/min, 1000cc/min O_2) and depth of anaesthesia was established by checking blinking reflexes and tail and foot withdrawal reflexes in all animals. Once anaesthetised, the chest cavity was opened and the ribcage removed to expose the heart. The right atrium of the heart was pierced using scissors or a large gauge syringe needle until blood was observed. A 25 gauge syringe needle (Terumo 26 x 5/8”) connected to a 60 mL syringe filled with ice cold 0.1M PBS was placed into the left ventricle of the heart and the PBS was washed through at a rate of 2mL/minute. Systemic blood washout was considered successful when the liver lightened in colour, and death confirmed with the cessation of breathing and cardiac arrest and then the animal was perfused with 4% PFA (approx.. 60 mL) until the body was rigid. The brain was then removed and fixation was deemed successful if the brain was a pale white colour. Brains were then stored in 4% PFA at 4℃ until further use.

2.3.6.2. Sectioning

One day prior to immunohistochemistry experiments, the brains were transferred to a 30% sucrose solution in 0.1 M TBS overnight. The following day, the brains were sectioned into 40 µm slices in cold 0.1M PBS using a Vibratome. Rat brain regions (prefrontal cortex: coronal sections, bregma +1.94 mm; ventral hippocampus, horizontal sections, bregma -3.08 mm) were determined using a rat

brain atlas. Slices were transferred to a 24 well plate containing 0.1M PBS with 1% sodium azide before immunolabelling.

2.3.6.3. Immunolabelling for Li-Cor visualisation

Slices were incubated for 1 hour at room temperature in blocking solution (0.1M TBS, 5% normal donkey serum (Sigma), 0.1% TX-100). Slices were then washed 3 times (0.1M TBS, 0.3% TX-100) and incubated on a shaker overnight at 4℃ with the primary antibodies: Rabbit anti-GluA1 (1:1000, Millipore) or rabbit anti GluA1 PhosphoSer845 (1:1000 Millipore) in 0.1M TBS with 2.5% normal donkey serum and 0.3% TX-100. The following day, the slices were washed 4 times every 15 minutes with TBST (0.1M TBS and 0.05% Tween 20), then incubated at room temperature for 1-2 hours with the secondary antibodies raised against that of the primary antibodies: donkey anti-rabbit (1:1000, Li-Cor) in 0.1M TBS with 2.5% normal donkey serum and 0.1% TX-100. Following four washes with TBST, slices were placed in 0.1M TBS before being mounted onto microscope slides, washed with distilled water and air dried. Slides were then sealed with mounting medium (Vectashield) and sealed for future analysis. According to the manufacturer, slices are viable for scanning for up to a year stored at 4℃, but slices were scanned usually 24 hours after being mounted.

2.3.6.4. Immunoblotting for confocal visualisation

Slices were incubated for 1 hour at room temperature in blocking solution (0.1M TBS, 5% normal goat serum (Sigma), 0.1% TX-100). Slices were then washed 3 times (0.1M TBS, 0.3% TX-100) and incubated on a shaker overnight at

4℃ with the primary antibodies: Rabbit anti-GluA1 (1:500, Millipore), or rabbit anti-GluA1 PhosphoSer845 (1:500 Millipore) co-labelled with mouse anti-PSD-95 (1:200, Abcam) in 0.1M TBS with 2.5% donkey serum and 0.3% TX-100. The following day, the slices were washed 4 times every 15 minutes with TBST (0.1M TBS and 0.05% Tween 20), then incubated at room temperature for 1-2 hours with the secondary antibodies raised against that of the primary antibodies: Alexa red 568-tagged goat anti-rabbit (1:500, Life) and Alexa 488-tagged goat anti-mouse (1:500, Life). Following four washes with TBST, slices were rinsed in 0.1M TBS before being mounted onto microscope slides with mounting medium (Vectashield Hardmount), stored at 4℃ and visualised 24-48 hour s after staining.

2.3.6.5. Li-Cor visualisation

Slides were scanned using a Li-Cor Odyssey Clx infrared scanner at 21 µm resolution at 700 nm wavelength. The detailed analysis of images is explained in Appendix B. An area outside of the brain slice was assigned as zero and regions of interest were highlighted for signal intensity measurements. These measurements were divided by the size of the highlighted area in pixels, giving a signal/pixel measurement. Background signal was subtracted Data were analysed by two-way ANOVA with Bonferroni post-hoc analysis compared to saline control.

2.3.6.6. Confocal visualisation

A confocal imaging system (Leica LSM880) with a x63 oil immersion lens was used for image acquisition. The detailed description of this method is in Appendix B. Each image was collected by averaging 5 scans at a resolution of 1024x1024

pixels. Regions of interest (dendritic layers of the CA1, CA3 and dentate gyrus) were scanned by lasers at 568 (GluA1 and GluA1 phosphoSer845, red), 488nm (PSD95, green) and 560nm (DAPI, blue). Slices were stained in duplicate and each slice was imaged in three different sites of each region of interest. To control for variability in brightness and contrast between images, all measurements were expressed in terms of ratios. All measurements were made using Fuji ImageJ and the colocalisation of the GluA1 signal with the PSD95 signal was used to normalise signals between slices. The Pearson's R value using Costes regression analysis was used as a measure of protein colocalisation; where a value of -1 indicated a total lack of protein colocalisation 1 a total protein colocalisation. Dendritic regions not containing cell bodies (identified by the absence of DAPI staining) were selected for quantification and the means of between 3-7 images was used. Data were analysed by two-way ANOVA with Bonferroni post-hoc analysis for statistical significance and expressed as mean ± SEM.

2.4. Results

2.4.1. Acquisition and extinction of heroin CPP

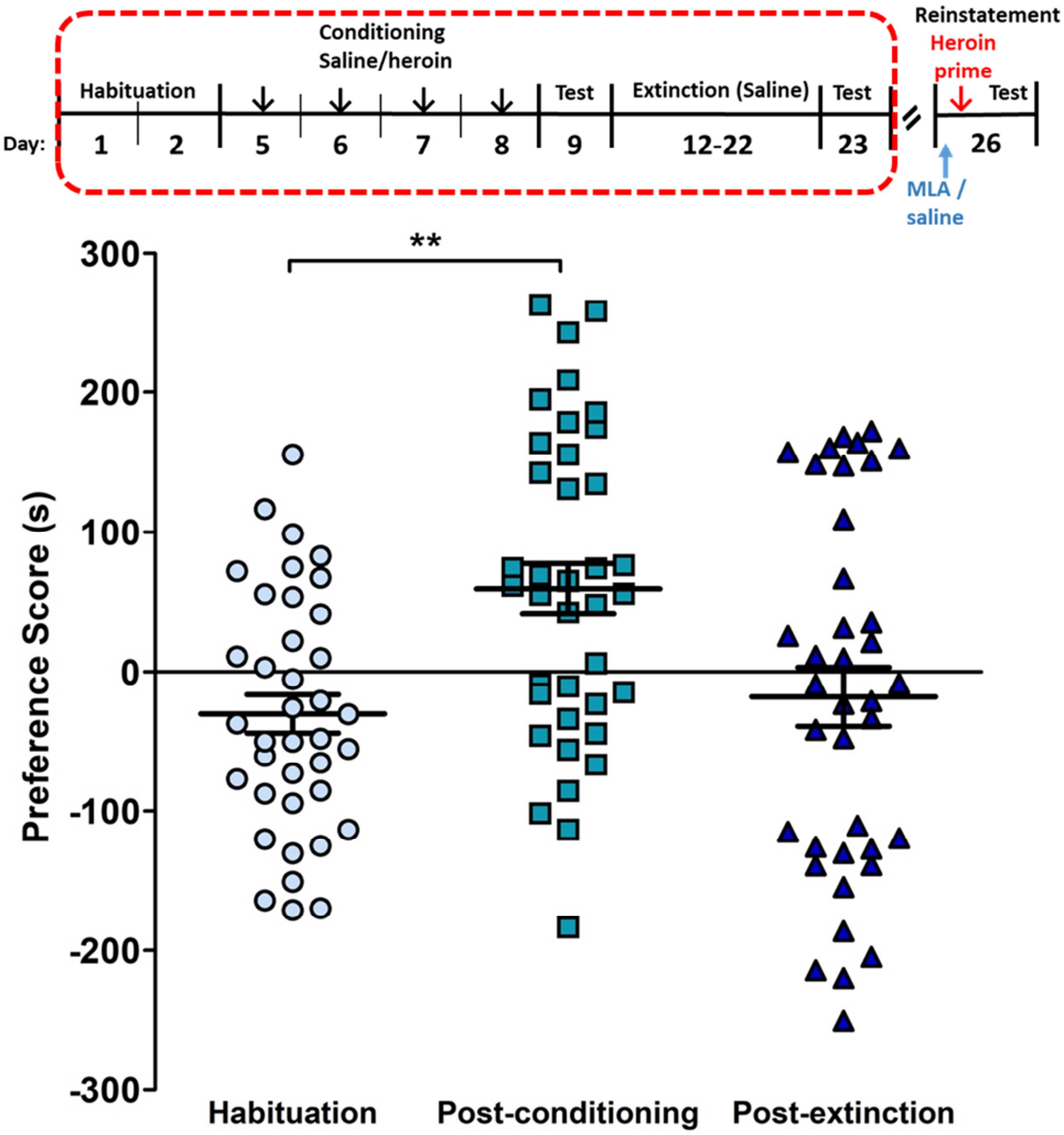


Figure 2.4: Preference scores for the habituation (mean of days 1 and 2), post-conditioning test (day 9) and post-extinction test (day 23). Data points are individual rat responses with mean±SEM overlaid. One-way ANOVA with Bonferroni post-hoc analysis vs habituation **p<0.01, n=38.

The initial aim of this study was to validate heroin CPP in rats (Figure 2.4). During habituation, on average, the rats showed no preference for the drug-paired

chamber. Preference for the drug paired side appeared evenly distributed among rats, suggesting effective counterbalancing of the designated drug-paired chamber. In the post-conditioning test, rats spent on average 95 seconds longer in the drug-paired side compared to habituation; demonstrating a significant increase in preference for the drug-paired side. The spread of the post-conditioning preference scores showed that although this dose of heroin was effective in producing CPP, it was perhaps not optimal. This said, 63% of all rats showed a positive preference score, and 54% of these positively scoring rats showed robust preference (>100 seconds). There were still a proportion of rats (37% of total) that appeared to show aversion for the drug-paired side (negative preference values). Importantly however, these were the rats that showed a negative preference score during the post-conditioning test, but there was still an overall increase in preference relative to their habituation scores, therefore they were kept in the study. During the post-extinction test, the mean preference score was significantly reduced compared to habituation. There was no difference between mean post-extinction and habituation preference scores. Again, the preferences were spread over a range of positive and negative scores, though the overall trend was a decrease in preference relative to the post-conditioning test, demonstrating a relative decrease in preference for the drug-paired side. The rats that showed the lowest negative scores were those that showed negative scores during habituation.

Overall, these initial results showed that the conditioning dose of 1mg/kg heroin was effective in producing CPP in rats. Importantly, the CPP was not too robust, as the behaviour was extinguishable, an important factor for the study of

reinstatement. As the CPP was deemed successful, rats were progressed onto reinstatement, to study the effects of antagonising α7nAChRs on this behaviour.

2.4.2. Pseudo-random assignment of treatment groups

Following extinction, rats were pseudo-randomly assigned to one of four treatment groups: saline control (S+S), heroin reinstatement (S+H), MLA control (MLA+S) and MLA reinstatement (MLA+H), where they received either saline (S) or MLA 20 minutes prior to a saline prime (S) or heroin prime (H) (see Figure 2.3 in methods section 2.3.5.7 for further detail). The study was pseudo-randomised by ensuring the preference scores observed during the habituation test, post-conditioning and post-extinction tests were all balanced, as demonstrated in **Figure 2.5** below.

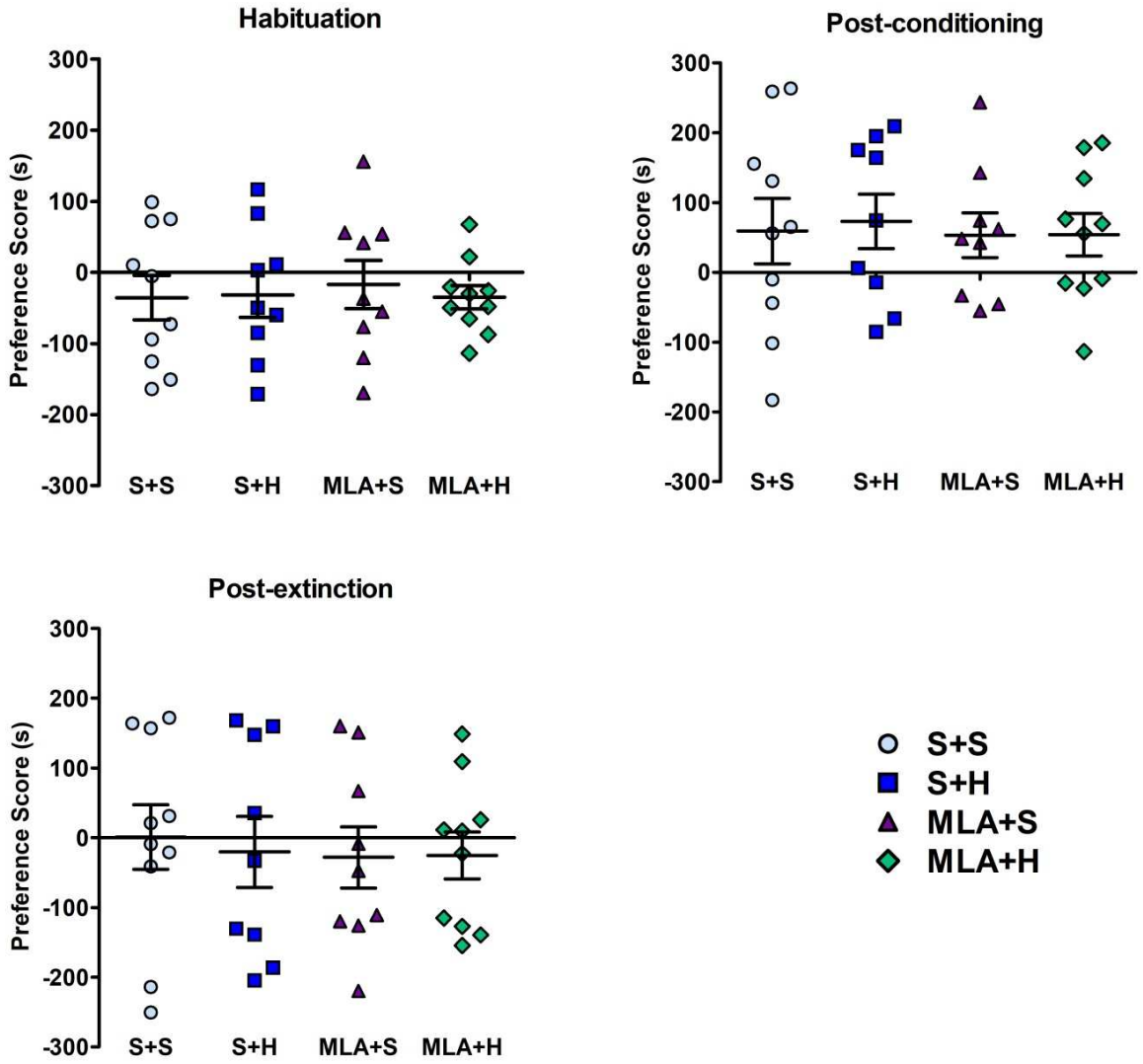


Figure 2.5: Comparison of preference scores during habituation, post-conditioning and post-extinction tests for the pseudo-random assignment of treatment groups for reinstatement. Data points are individual rat responses with mean±SEM overlaid. Two-way ANOVA showed no difference between assigned groups. S+S: saline control group: n=10; heroin reinstatement group: S+H n=9; MLA control group: MLA+S n=9; MLA reinstatement group: MLA+H n=10.

Figure 2.5 shows the habituation, post-extinction and post-conditioning preference scores for rats assigned to treatment groups. Two-way ANOVA analysis showed no difference in the preference scores between treatment groups, and the data

was evenly spread between treatment groups, suggesting the study was correctly balanced.

To date, only 2 studies have investigated heroin-primed reinstatement of heroin CPP (to my knowledge) (Leri and Rizos, 2005, van der Kam et al., 2009). van der Kam et al. (2009) found that 0.25mg/kg heroin significantly reinstated heroin CPP in rats conditioned with 0.25mg/kg heroin. However, systematic review of this dose showed that it did not always produce CPP in rats (Bardo et al., 1995, Tzschentke, 2007). Leri and Rizos (2005) tested 2 different priming doses of heroin (0.3 and 1 mg/kg, s.c.) to reinstate CPP in rats conditioned with 1 mg/kg heroin. They found that only the dose of 1 mg/kg heroin produced significant reinstatement of CPP. Another study supporting this priming dose found that 1 mg/kg heroin caused the significant reinstatement of CPP in rats conditioned with morphine (Lu et al., 2002). The heroin priming dose of 1 mg/kg was therefore chosen for this study and the effect of antagonising α7nAChRs on reinstatement was investigated.

2.4.3. Effect of MLA on heroin-primed reinstatement of CPP

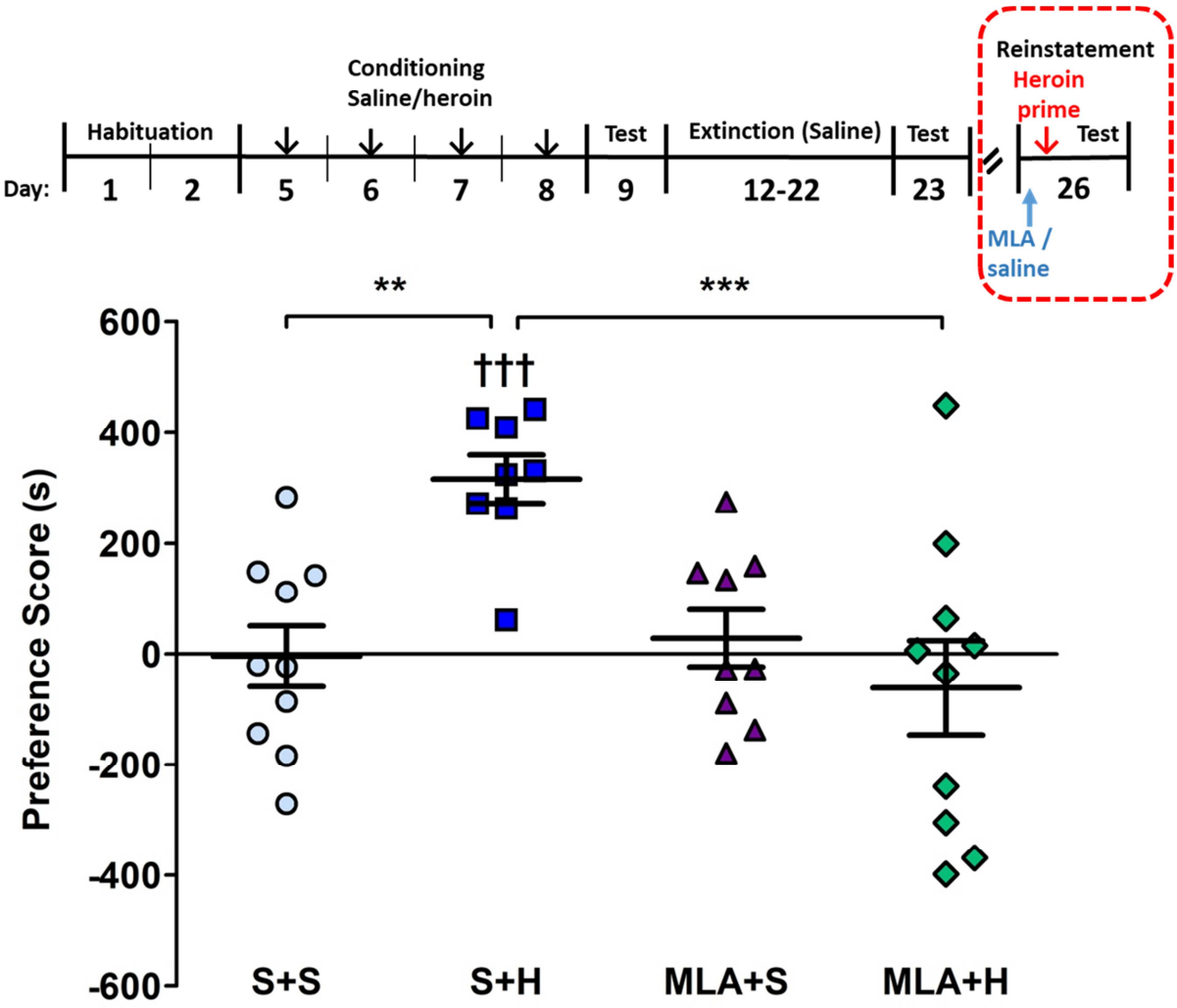


Figure 2.6: Effect of MLA on heroin-primed reinstatement of CPP. Treatment groups were saline control (S+S, n=10), heroin reinstatement group (S+H, a Grubb's outlier test showed one rat to be an outlier, therefore n=8 for reinstatement), MLA control group (MLA+S n=9) and MLA reinstatement (MLA+H n=10). Data points are individual rat responses with mean±SEM overlaid. Two-way ANOVA with Bonferroni post-hoc analysis vs post-extinction $^{\dagger\dagger\dagger}p<0.001$; vs S+H. $^{**}p<0.005$, $^{***}p<0.001$.

The saline control group (S+S, **Figure 2.6**) showed no increase in preference for the drug-paired side compared to saline extinction, showing no effect of saline priming on reinstatement. In the heroin reinstatement group (S+H) there was a significant increase in the time spent in the drug-paired compartment compared to post-extinction, and compared to the saline control group. This demonstrated a

significant reinstatement of drug seeking behaviour in this treatment group. Interestingly, the reinstatement response was four times greater than the post-conditioning preference scores, suggesting a robust recall of the drug-related behaviour. MLA pre-treatment had no effect on the reinstatement of CPP in saline-primed animals, but significantly inhibited heroin-primed reinstatement of CPP (MLA+H).

These results show that the selected priming dose of heroin (1 mg/kg, s.c.) was effective in reinstating CPP. They also demonstrate that MLA alone has no reinforcing or aversive properties as it failed to induce reinstatement. MLA pre-treatment was effective in significantly inhibiting reinstatement of heroin-primed CPP. This inhibition of reinstatement by MLA could be explained by MLA blocking the rewarding properties of heroin and therefore reducing the motivation to seek the heroin-paired chamber. To test this, the effect of MLA was investigated on the acquisition of CPP.

2.4.4. Effect of MLA on the acquisition of heroin CPP.

As the inhibition of heroin-primed reinstatement by MLA could be explained by the decrease in the rewarding effects of heroin, the effect of MLA on the acquisition of heroin CPP was tested. A new group of rats was used for this study (see methods section 2.3.5.3). Briefly, after habituation, rats (n=24) were pseudo-randomly allocated a treatment group, and received either saline (S+H, 1 mL/kg saline s.c.) or MLA (MLA+H, 4 mg/kg s.c.) 20 minutes prior to conditioning, where they received a heroin injection (1 mg/kg s.c.) and were confined to their assigned drug-paired side or a saline injection (1 mL/kg, s.c.) and were confined to the

unpaired side for four consecutive days. MLA was administered before both heroin and saline conditioning days. On the fifth day they were placed free-roaming in the chamber for 15 minutes in the post-conditioning test.

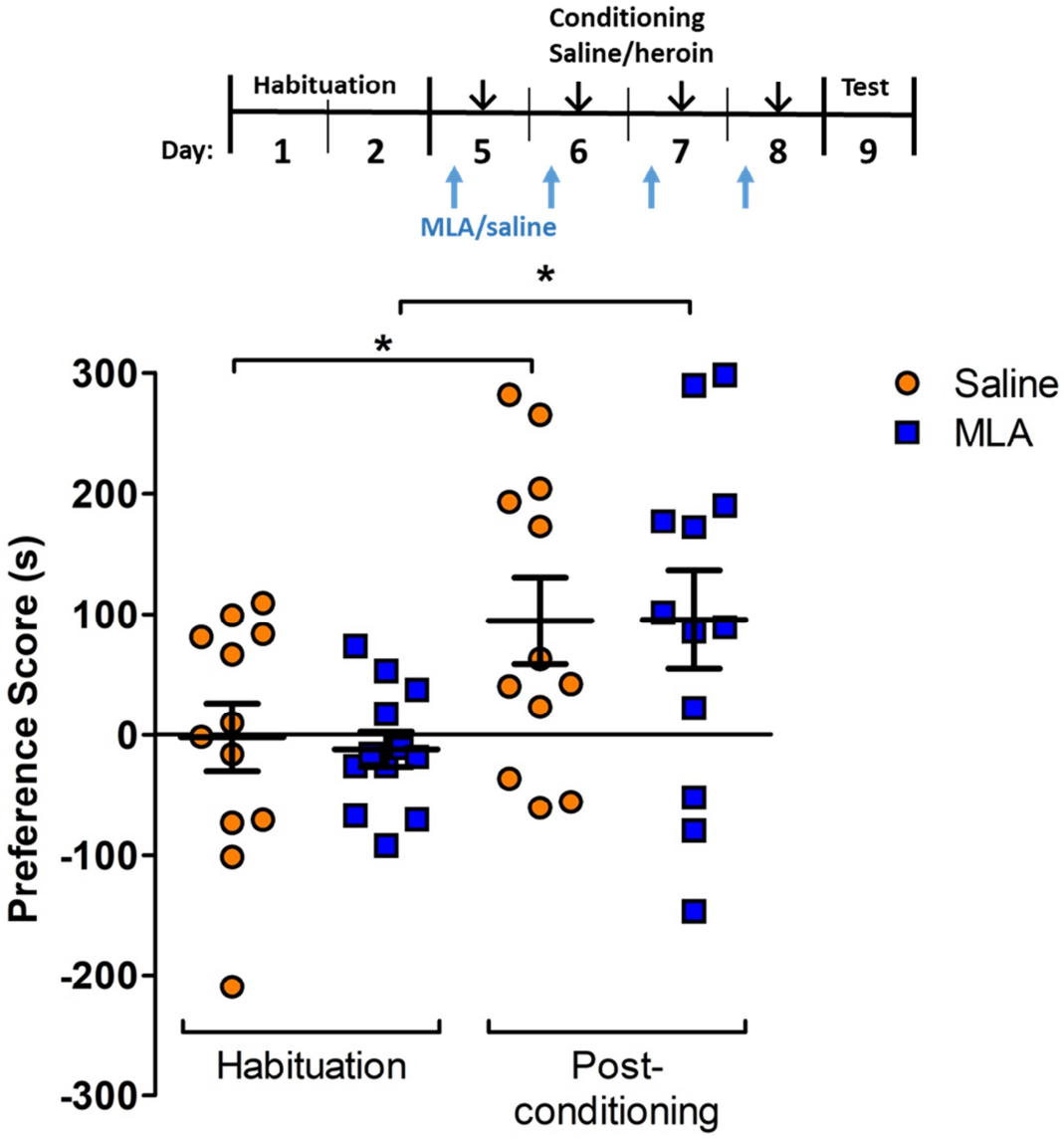


Figure 2.7: Effect of MLA pre-treatment on the acquisition of heroin CPP. Rats underwent 2 habituation sessions (drug-free) and were then pseudo-randomly assigned a treatment group: Saline-pre-treatment (orange circles) or MLA (4 mg/kg, s.c.) pre-treatment (blue squares) 20 minutes prior to all conditioning sessions. Data points are individual rat responses with mean±SEM overlaid. Paired t test habituation vs post-conditioning for each treatment group *p<0.05, S+H n=12, MLA+H n=12.

All rats underwent habituation and were then pseudo-randomly assigned a treatment group so there was no difference in habituation preference scores (Figure 2.7). In the post-conditioning test, both the saline and MLA pre-treatment groups showed a significant increase in preference for the drug-paired side compared to their respective habituation scores. Importantly, there was no difference in the post-conditioning preference scores between both treatment groups, demonstrating that MLA had no effect on the acquisition of heroin CPP. These results, combined with the previous section (2.4.3) demonstrated that MLA did not inhibit the heroin-primed reinstatement of CPP by reducing the rewarding effects of heroin.

2.4.5. Effect of MLA on locomotion during heroin CPP

2.4.5.1. Habituation, extinction and acquisition

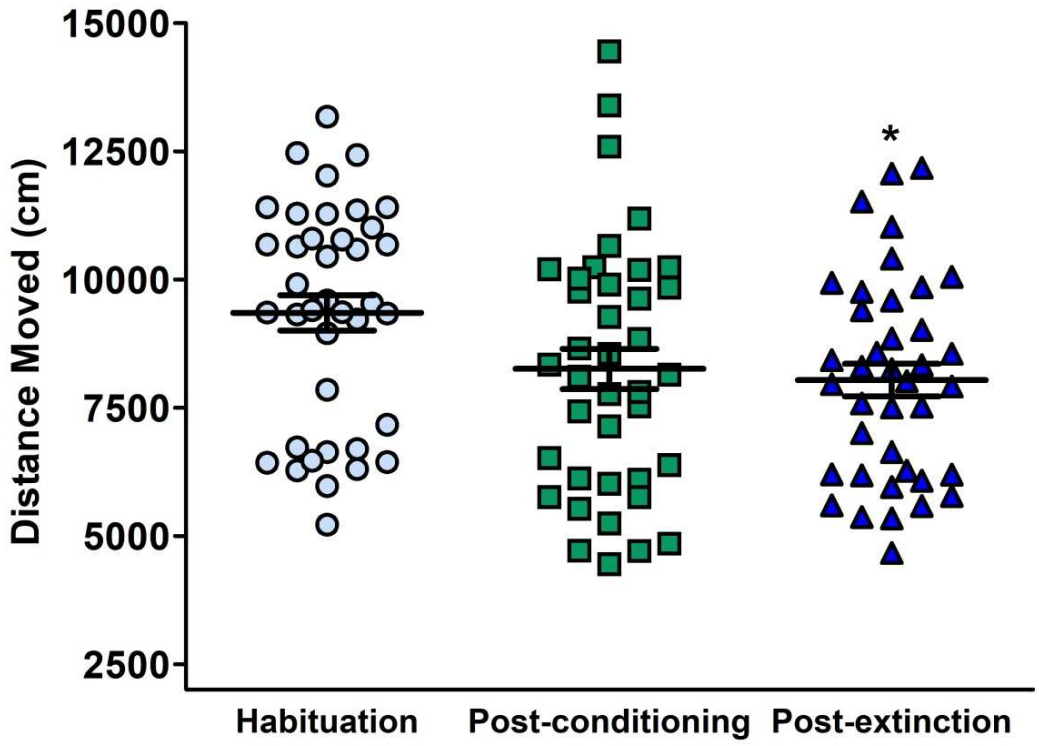


Figure 2.8: Total distance moved (cm) during habituation, post-conditioning and post-extinction tests. Data points are individual rat responses with mean±SEM overlaid. One-way ANOVA with Bonferroni post-hoc analysis vs habituation *p<0.05; n=38.

The habituation, post-conditioning and post-extinction tests were all carried out in a drug-free state. There was a slight decrease (by 10%) in the distance travelled in the post-conditioning test, compared to habituation (Figure 2.8). There was a significant 14% decrease in the distance moved during the post-extinction phase compared to habituation.

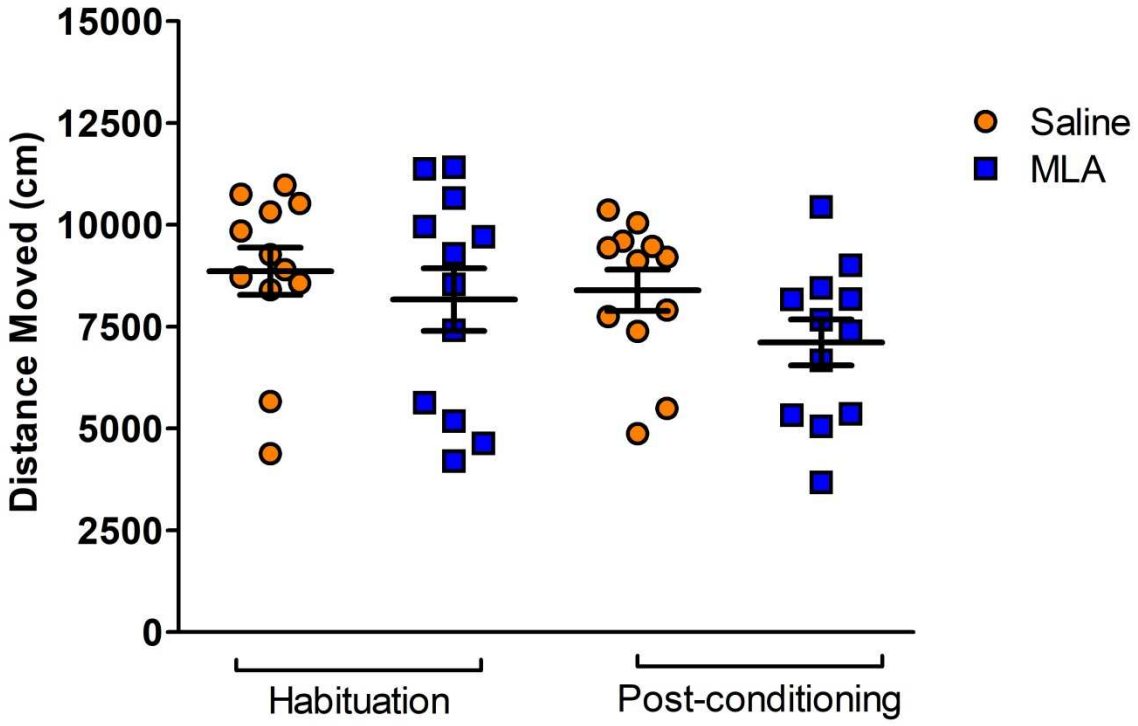


Figure 2.9: Effect of MLA pre-treatment on the distance moved during the acquisition (post-conditioning) of heroin CPP. Rats underwent 2 habituation sessions (drug-free) and were then pseudo-randomly assigned a treatment group: Saline-pre-treatment (orange circles) or MLA (4 mg/kg, s.c.) pre-treatment (blue squares) 20 minutes prior to all conditioning sessions. Data points are individual rat responses with mean±SEM overlaid. Paired t test, n=12 in each group.

In rats that received either saline or MLA pre-treatment during the heroin conditioning phase (Figure 2.9), there was no difference in locomotion between both treatment groups during the post-conditioning test. Importantly, the post-test was administered in a drug-free state; therefore it cannot be determined whether MLA had any effect at this stage. It appears that there were no long-term effects of MLA administration during conditioning on locomotion on the post-conditioning test.

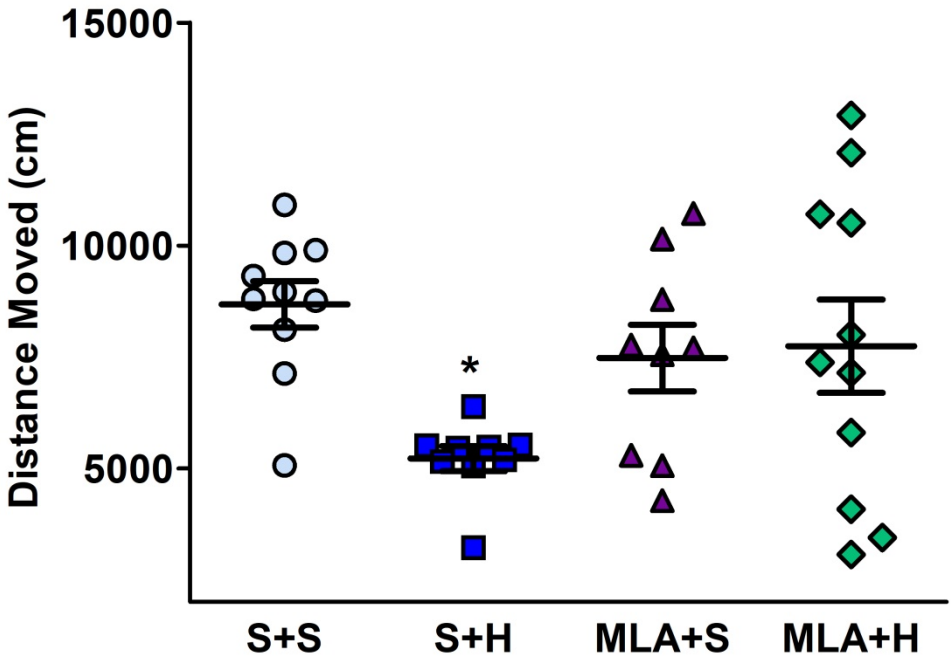


Figure 2.10: Total distance moved during heroin-primed reinstatement of CPP. Treatment groups are saline (S+S, n=10), heroin reinstatement group (S+H n=8), MLA control group (MLA+S n=9) and MLA pre-treatment on heroin reinstatement (MLA+H n=10). Data points are individual rat responses with mean±SEM overlaid. One-way ANOVA with Bonferroni post-hoc analysis vs S+S *p<0.05.

During the reinstatement phase, the fact that some rats were in a drugged state (S+H and MLA+H) and others were undrugged (S+S and MLA+S) must be considered when comparing their distance travelled. There was no difference in the distance travelled between the saline control (S+S, Figure 2.10) and MLA control (MLA+S) groups, suggesting a lack of effect of MLA on this behaviour. There was however a significant decrease in locomotion in the heroin reinstatement group (S+H, Figure 2.10) compared to MLA reinstatement (MLA+H), suggesting a potential effect of heroin on the locomotion of the animals. The interesting effect was that the MLA reinstatement group (MLA+H) showed no difference in distance travelled compared to saline control and MLA control, suggesting a potential reversal of the effect of heroin on the distance moved

during reinstatement. The reduction in locomotion during heroin-primed reinstatement could potentially be explained by the drug conditioned memory being re-activated, leading animals to travel directly to the drug-paired side and remain in this compartment. MLA potentially blocks this re-activation of the drug-associated memory; therefore animals spend more time exploring the apparatus. This was investigated by measuring the number of crossovers between CPP compartments.

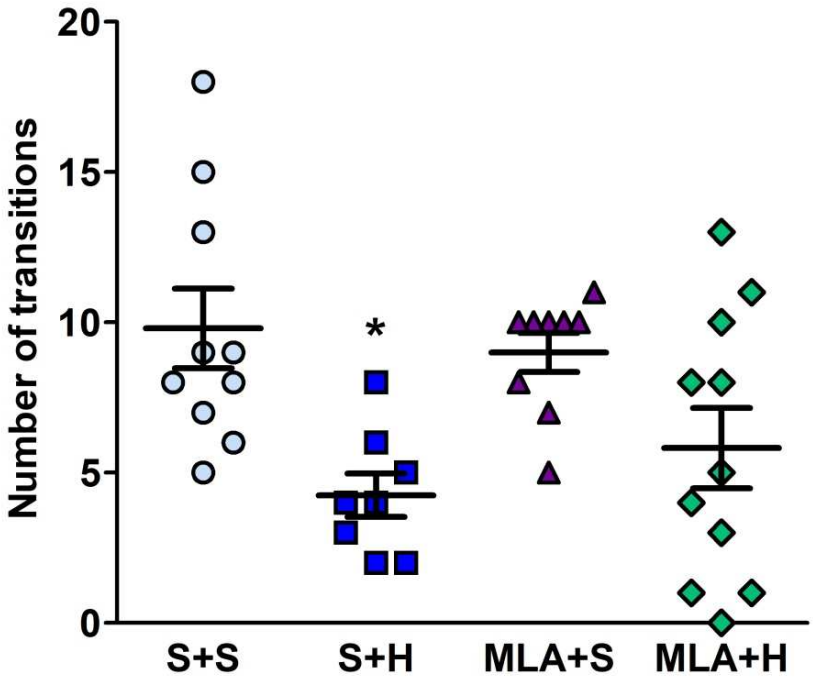


Figure 2.11: Number of zone transitions during reinstatement. Treatment groups are saline (S+S, n=10), heroin reinstatement group (S+H n=8), MLA control group (MLA+S n=9) and MLA pre-treatment on heroin reinstatement (MLA+H n=10). Data points are individual rat responses with mean±SEM overlaid. One-way ANOVA with Bonferroni post-hoc analysis comparing S+H vs MLA+H *p<0.05

When analysing the number of transitions between CPP compartments, there was a significant decrease in transitions in the heroin reinstatement group (S+H, Figure 2.11) compared to the saline control group (S+S). This suggests a reduction in the

exploration of the CPP apparatus. There was no effect of MLA alone on the number of transitions. In the MLA reinstatement group (MLA+H), there was no difference in the number of transitions compared to saline (S+S), suggesting a potential reversal of the effect seen in the S+H group; however there was also no significant difference compared to the heroin reinstatement group, mostly due to the increased variability observed in the MLA+H group.

2.4.6. Quantification of AMPA receptors

Following reinstatement, rats were immediately perfused with 4% paraformaldehyde (PFA) under terminal anaesthesia (inhalable isoflurane, 2-3 cc/min, 1000cc/min O_2) to fix the brain tissue (see methods section 2.3.6.1). Brains were then sliced and immunolabelled for GluA1 AMPA receptor subunits, as these are shown to be increased at the synapse during LTP (Citri and Malenka, 2008, Henley and Wilkinson, 2013) and GluA1 AMPA receptor subunits phosphorylated at Ser845 as these are shown to be involved in the priming of AMPA receptors for LTP (increase channel open probability Roche et al. (1996)) and to be involved in the trafficking of AMPAR to the synapse (Roche et al., 1996, Oh et al., 2006, Derkach et al., 2007). Brains were then probed with the appropriate secondary antibodies for visualisation by either Li-Cor near-infrared scanning technology or confocal microscopy (details in methods section 2.3.6.3 and 2.3.6.4, respectively). The optimisation of both methods is described in Appendix B. Each brain slice was visualised by both methods to allow a comparison of the sensitivity of the visualisation method and to probe whether both methods would detect comparable changes in AMPA receptor expression.

2.4.6.1. Li-Cor quantification of AMPA receptor subunits

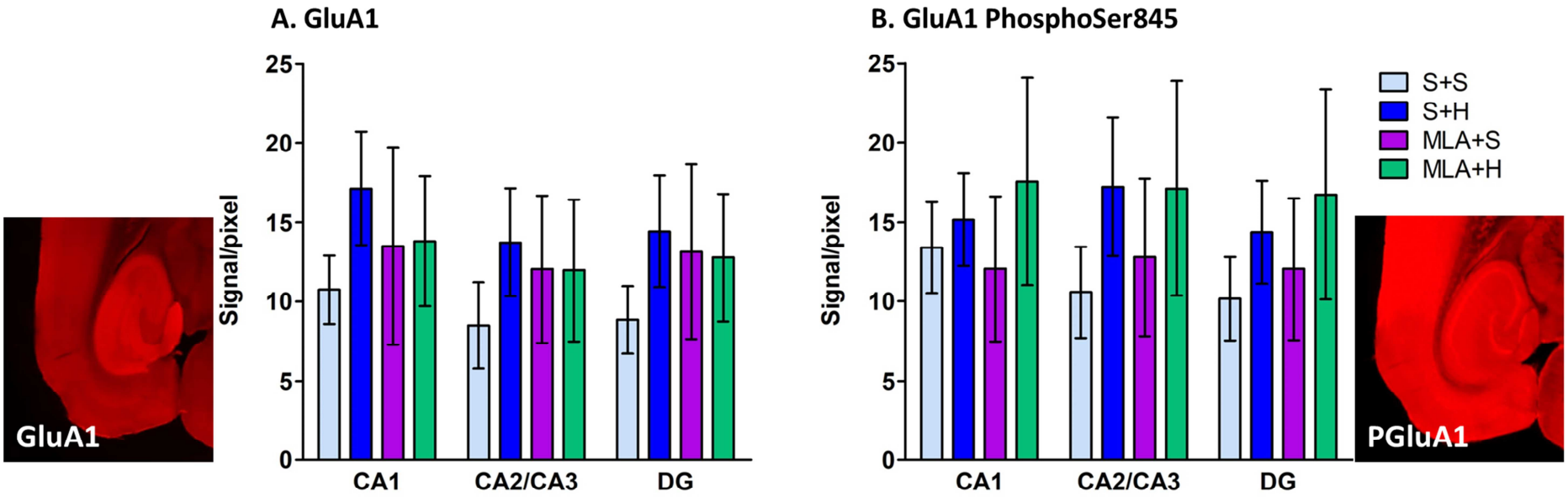


Figure 2.12: Quantification of GluA1 and representative visualisation of GluA1 **(A)** and GluA1 phosphoSer845 **(B)** in the CA1, CA2/CA3 and dentate gyrus (DG**)**. Images were scanned on a Li-Cor Odyssey Clx scanner at 700nm at 21µm resolution. Two-way ANOVA, n=5 rats per treatment group

Whole brain slice quantification of GluA1 subunits (Figure 2.12A) by Li-Cor near-infrared scanning technology in the ventral hippocampus showed the lowest levels of GluA1 expression were in the saline control group (S+S), through all 3 regions of the ventral hippocampus. In the heroin reinstatement group (S+H) there was a 1.7-fold increase in GluA1 expression in the CA1, and a 1.6-fold increase in the CA2/CA3 and dentate gyrus, compared to saline control. There wasno significant change in GluA1 expression in the MLA control (MLA+S) and MLA reinstatement (MLA+H) groups compared to heroin reinstatement (S+H) due to large variability.

GluA1 phosphoSer845 expression in the ventral hippocampus (Figure 2.12B) was also the lowest in the saline control group and remained consistent across all three regions of the ventral hippocampus. There were similar trends of expression were observed in all three regions between treatment groups. There was a slight increase in expression in the heroin reinstatement group (S+H) and the MLA reinstatement group (MLA+H), and a decrease in the MLA control group (MLA+S). Levels in the MLA reinstatement group (MLA+H) however were the highest, but due to large variability, there was no significant difference between all treatment groups.

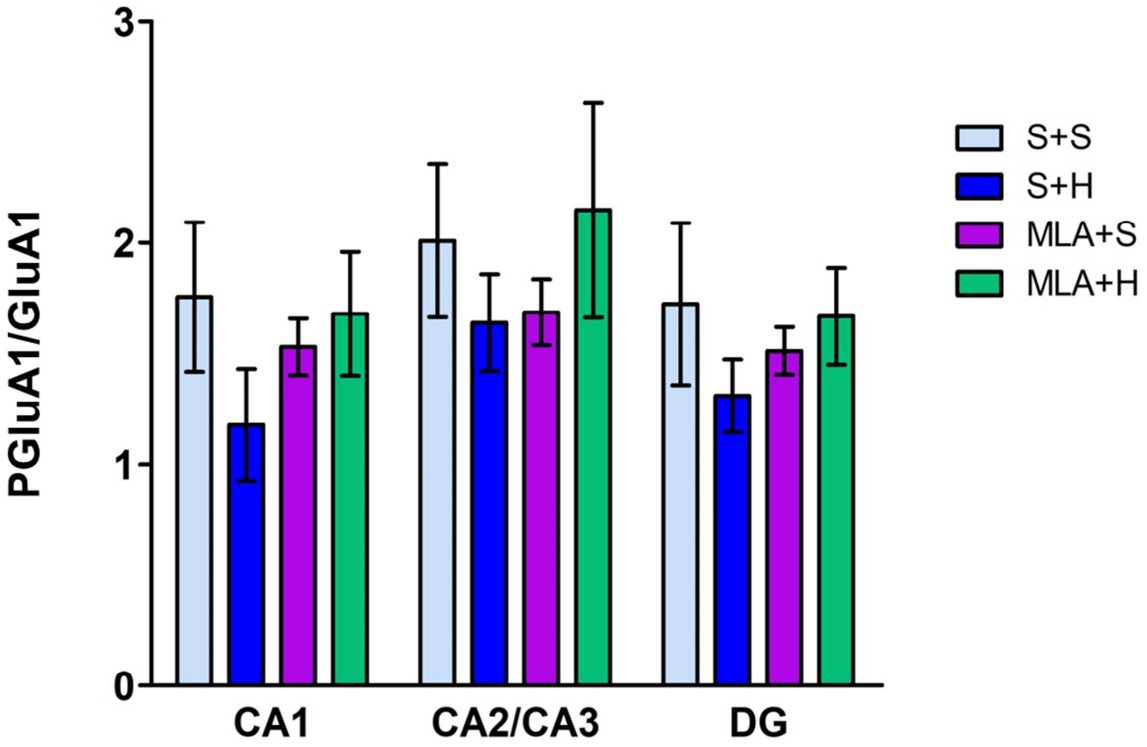


Figure 2.13: Phosphorylated GluA1 signal normalised to GluA1. Two-way ANOVA, n=5 rats per treatment group.

GluA1 PhosphoSer845 signals normalised to GluA1 (**Figure 2.13**) allowed the reduction of the variability in signal intensity between slices. In the CA1, in heroin-primed animals, there was a ~30% decrease in phosphorylated GluA1 compared to saline control, though there was no significant difference. There was no change in phosphorylated GluA1 in the MLA control group or in the MLA reinstatement group. In the CA2/CA3 and dentate gyrus, there appeared to be a similar trend, but the MLA control group showed similar expression of phosphorylated GluA1 to the heroin reinstatement group. There appeared to be a trend of was a slight decrease in phosphorylated GluA1 in the heroin reinstatement group (S+H) in the CA1 by 30%, and in the CA2/CA3 and dentate gyrus, to a lesser extent (20%). Again, due to variability, these results were not significant.

There were consistent levels of GluA1 and GluA1 PhosphoSer845 across the regions of the ventral hippocampus in each treatment group, suggesting either a certain degree of consistency of the quantification method or a lack of sensitivity. Using the Li-Cor quantification method, there was no detectable effect of heroin-priming or of MLA pre-treatment on GluA1 or GluA1 phophoSer845 expression in either the CA1, CA2/CA3 or dentate gyrus regions of the ventral hippocampus. To address the issue of a potential lack of sensitivity of this method, and to identify and quantify AMPA receptor subunits at the postsynaptic membrane, slices were also scanned using confocal microscopy. This would allow a direct comparison of the results obtained in either visualisation method.

2.4.6.2. Confocal quantification of AMPA receptor subunits

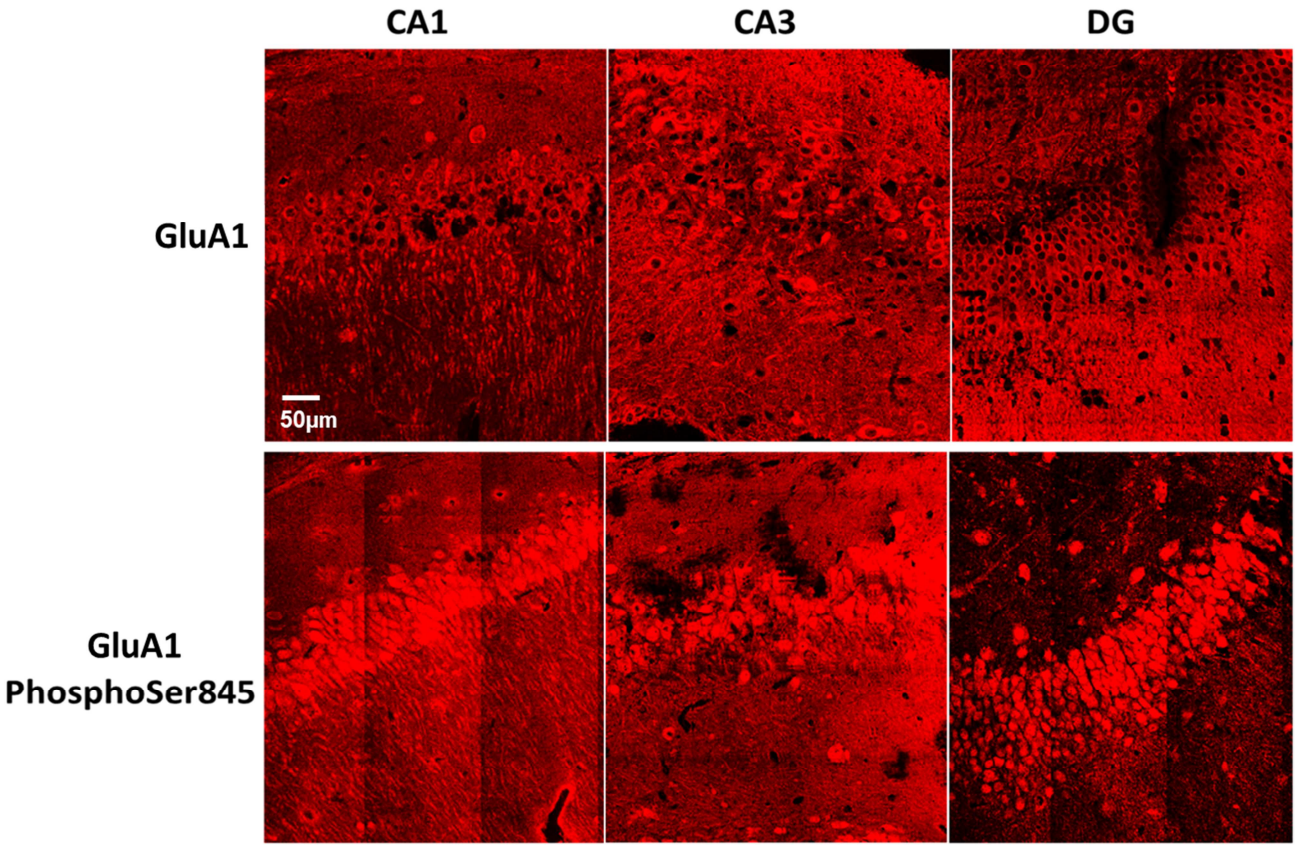


Figure 2.14: Representative tile scan of the CA1 **(left)**, CA3 **(middle)** and dentate gyrus (DG, **right**) regions of the ventral hippocampus probed for GluA1 AMPA receptor subunits (top) and GluA1 phosphoSer845 AMPA receptor subunits **(bottom)**. 5x5 image tile scan at 63x magnification with oil immersion.

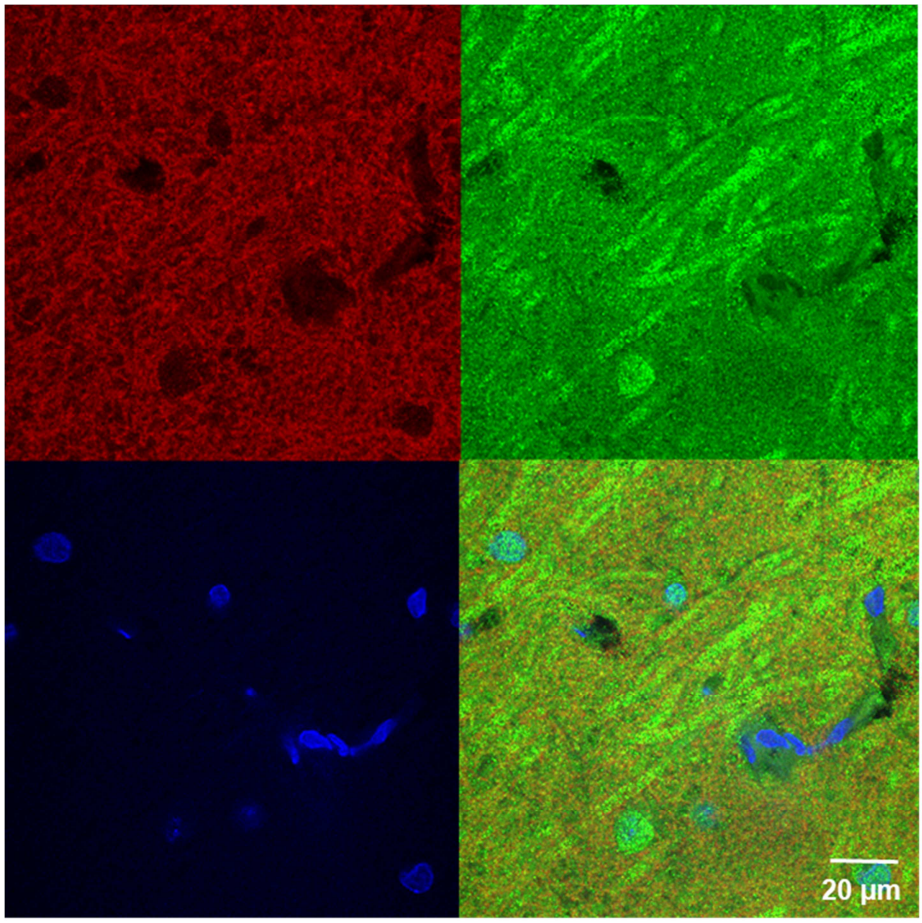


Figure 2.15: Representative image of the highlighted dendritic layer of the CA1 region of the ventral hippocampus, for GluA1 (red) and PSD95 (green) quantification. DAPI nucleus stains are in blue. 63x magnification with oil immersion.

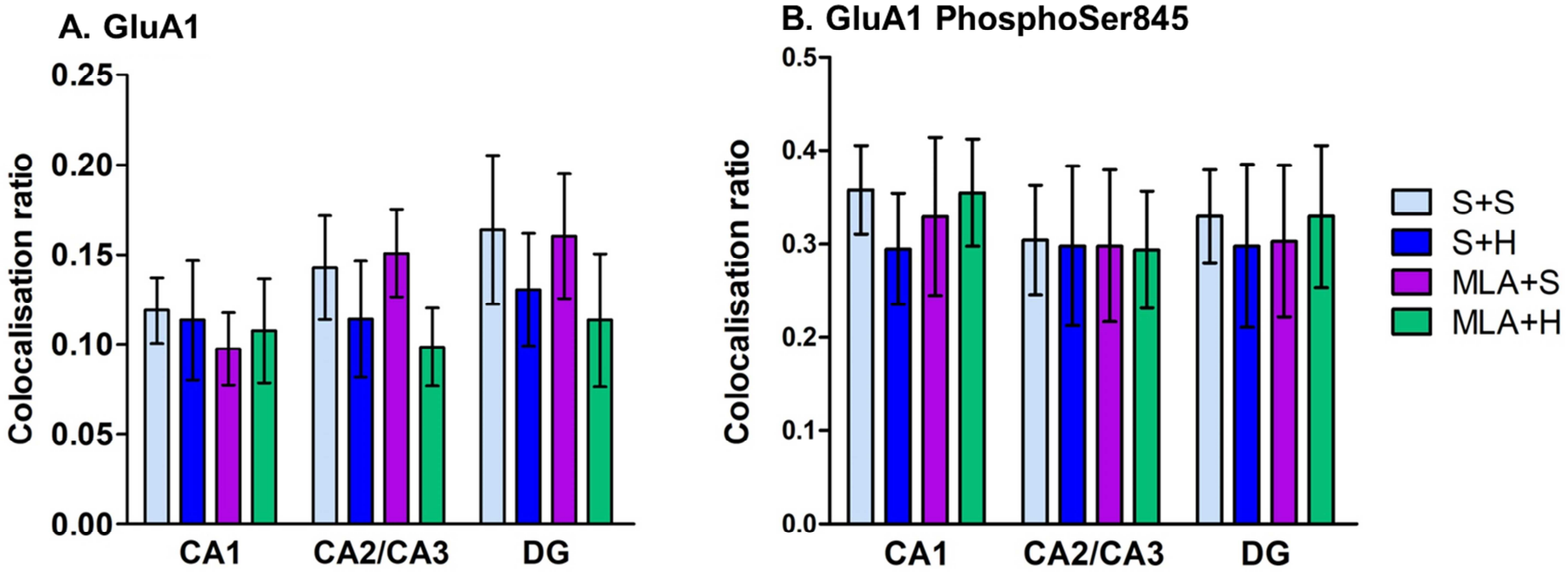


Figure 2.16: Quantification of GluA1 **(A)** and GluA1 phosphoSer845 **(B)**-containing AMPA receptors using confocal microscopy on rat brains from heroin-primed reinstatement of CPP experiments. Regions of interest were the CA1, CA2/CA3 and dentate gyrus (DG) regions of the ventral hippocampus. Two-way ANOVA, n=5 per group.

Figures 2.14 and 2.15 show representative confocal images of regions of the hippocampus and dendritic layers visualised for quantification. Quantification of colocalisation ratios of GluA1 or GluA1 phosphoSer845 with PSD95 visualised by confocal microscopy (Figure 2.16) revealed a large amount of variability in the data. Analysis of GluA1 expression (Figure 2.16A) showed no change in expression in the CA1 region between treatment groups. In the CA2/CA3 and dentate gyrus, there was no difference in GluA1 expression in either the saline control (S+S) or MLA control (MLA+S) groups. There appeared to be a slight decrease In GluA1 expression in in the heroin and MLA reinstatement groups (S+H and MLA+H, **Figure 2.16**A), however due to variability there was no statistical difference between these groups.

There was no difference in GluA1 phosphoSer845 expression between all ventral hippocampus regions and between all treatment groups, except for a small decrease in the CA1 in the saline reinstatement group (Figure 2.16B, not significant). Again, there was a wide variability in signals.

Overall, with the confocal colocalisation quantification method, there appeared to be no effect of effect of heroin priming or of MLA pre-treatment on the expression of GluA1 or phosphorylated GluA1 subunits throughout the ventral hippocampus.

2.4.6.3. Comparison of visualisation techniques

There were no comparable trends in the expression of GluA1 or phosphorylated GluA1 between visualisation methods. In fact, in some instances, the opposite trend was observed. For example, Li-Cor visualisation showed a general increase in GluA1 expression in the heroin reinstatement group (S+H, Figure 2.12A),

whereas confocal visualisation showed a general decrease in expression of GluA1 in this same treatment group (S+H, Figure 2.16A). Furthermore, Li-Cor detection showed general increases of GluA1 phosphoSer845 expression in the heroin reinstatement and MLA reinstatement groups (S+H and MLA+H Figure 2.12B) compared to saline control, whereas confocal detection showed relatively consistent levels of expression throughout the ventral hippocampus and a slight decrease in expression in the heroin reinstatement group in the CA1 (S+H, Figure 2.16B). No significant difference in expression of GluA1 or phosphorylated GluA1 subunits was detected in either visualisation method, and the methods did not offer comparable results.

2.5. Discussion

2.5.1. Acquisition and extinction of heroin CPP

Heroin (1 mg/kg) was effective in producing CPP, as rats showed a significant increase in preference for the drug-paired side compared to habituation. Acquisition of CPP with this dose of heroin is comparable to findings by Leri and Rizos (2005), who used the same dose and the same route of administration (1 mg/kg, s.c.) in Long-Evans rats. In their 20-minute post-conditioning test, they found on average that rats spent roughly 42% of the total test time in the heroin-paired compartment, compared to 25% in the vehicle-paired side (there was a neutral compartment in this experiment as well). The habituation data was not shown in their publication. Other studies administered 1mg/kg heroin i.p. and found that rats spend approximately 50% (Tzschentke et al., 2006) and 60% (Galaj et al., 2015, Galaj et al., 2016) of total test time in the drug-paired side. In the present study, in the 15-minute post-conditioning test, rats spent 56% of total test time in the heroin-paired side, compared to 47% during habituation. These results show that the magnitude of CPP achieved in this study is also comparable with the findings of the studies mentioned above. Furthermore, these results are comparable with morphine CPP studies in rats and mice, which spent approximately 60% of the total test time in the drug-paired side in the post-conditioning test (Feng et al., 2011, Wright et al., 2018), and a meta-analysis of opioid CPP showed that morphine and heroin CPP preference scores in rats were of the same magnitude (Bardo et al., 1995). 1mg/kg heroin was shown to have the most robust effect on CPP, though studies have rarely studied doses above 1 mg/kg (Bardo et al., 1995). Heroin CPP in rats was therefore validated in this study.

During extinction, there was a significant reduction in preference for the drug-paired side, compared to the post-conditioning test. There was no difference compared to habituation, suggesting successful extinction of the conditioned behaviour. Interestingly, the extinction of heroin CPP required double the number of extinction sessions than morphine CPP, suggesting a stronger association with the drug-paired compartment.

Often, the priming dose used to trigger reinstatement is lower than the conditioning dose (Do Couto et al., 2003). In this instance, there was insufficient published evidence of lower doses of heroin reinstating CPP when, to my knowledge, only 3 studies were found to induce heroin-primed reinstatement of heroin (Leri and Rizos, 2005, van der Kam et al., 2009) and morphine (Lu et al., 2002) CPP. The heroin priming dose used by Leri and Rizos (2005) (1mg/kg, s.c.) was therefore based on this publication. In our study, the priming dose of heroin induced a significant reinstatement of heroin CPP, and interestingly, this preference was larger than during the post-conditioning test. On average, rats spent 56% of total test time in the drug-paired side during the post-conditioning test, whereas during reinstatement, they spent 86% of total test time. This effect was also reported by Leri and Rizos (2005), who found that rats spent 84% of total test time in the drug-paired side during reinstatement, compared to 42% during the post-conditioning test. The heroin-primed reinstatement of CPP in the present study are therefore in agreement with the findings by Leri and Rizos (2005). In morphine CPP studies, this effect of a larger reinstatement preference was also observed in mice and rats, but to a lesser extent than with heroin (Do Couto et al., 2003, Feng et al., 2011, Portugal et al., 2014, Wright et al., 2018). The reinstating effects of heroin can be attributed to the rewarding properties of the drug, which produces an

incentive motivational state that reinstates the preference. During CPP, the previously neutral CPP chamber acquires a salient value through repeated exposure to the drug reward. The reinstatement of drug-seeking behaviour can be explained as the recall of this salient value by the heroin priming dose, which leads the animal to return to spending more time in the drug-paired chamber. This approach behaviour could reflect a reinstatement of craving which drives the animal to seek the environmental cues associated with the euphoric effects of the drug. As rats were tested in a drugged state during reinstatement, it is possible that the presence of the heroin prime provides a stronger activation of the drug-associated memory (potentially by the recruitment of additional brain regions, see Chapter 1, section 1.3.4), which leads to the stronger preference response compared to the post-conditioning test.

2.5.2. MLA inhibits heroin-primed reinstatement of CPP

The key result from this study demonstrated that MLA blocked heroin-primed reinstatement of CPP. The data from this chapter extend the findings from our lab (Wright et al., 2018) and another study (Feng et al., 2011) showing that MLA is effective against reinstatement of morphine-induced CPP. Wright et al. (2018) however observed that systemic administration resulted in an attenuation of reinstatement of morphine CPP, whereas direct infusion into the ventral hippocampus resulted in the total inhibition of reinstatement. This is perhaps due to an increase in variability observed during reinstatement. In this thesis, the variability was greater in the MLA reinstatement group; however MLA treatment totally inhibited reinstatement (Figure 2.6). Here, we extend previous findings to show a similar effect against a more rewarding opioid, heroin. To our knowledge,

these are the only studies that have demonstrated the effects of MLA (or other α7 nAChR antagonists) on reinstatement to CPP induced by drugs of abuse other than nicotine itself.

Basal cholinergic activity appears crucial for cue-induced relapse as lesions of basal forebrain cholinergic neurons resulted in the significant reduction of discrete cue-induced reinstatement of cocaine seeking in rats (Section 1.4, Pitchers et al. (2017)), and α7nAChRs may mediate, at least in part, this cholinergic signalling.

The dose of MLA (4 mg/kg) was based on previous literature evidence for its selectivity and efficacy in mice and rats (Feng et al., 2011, Wright et al., 2018). It binds to the α-bungarotoxin binding site on α7nAChRs (Ward et al., 1990), however it has also been shown to bind to α3 and α6β2 subtypes at high concentrations (Mogg et al., 2002), though at the dose used in this study (4mg/kg), the brain plasma concentrations would be in the subnanomolar range (Nirogi et al., 2012). Chilton et al. (2004) reported that lower doses of MLA (1 and 3.2, but not 10 mg/kg) induced a reduction in locomotion in mice, though they were scored over a one hour period and the decrease in locomotion was reported as “sometimes present”. They did not however show the change in behaviour over time, which could have determined whether this decrease in locomotion was constant or transient during the testing period. There was no effect of MLA alone on the distance moved in the reinstatement session reported by Wright (2016) and in this thesis.

The 20 minute pre-incubation time was to allow sufficient MLA to reach the brain before heroin-priming, where maximum brain plasma levels were reported at 30 minutes after administration (Turek et al., 1995, Nirogi et al., 2011, Nirogi et al.,

2012), though this was done with oral and intravenous routes of administration and there is currently no literature on the pharmacokinetics and bioavailability of MLA by the subcutaneous route of administration. This time frame also did not allow enough time to inhibit object recognition memory reported by Tinsley et al. (2011) which was observed 24 hours after MLA administration at a very low dose of MLA (87.5µg/kg, i.p.), but not 20 minutes post-injection. This effect was however attributed to the perirhinal cortex. Furthermore, MLA had no effect on the acquisition of heroin CPP as the post-conditioning test was performed 24 hours after the final conditioning session. In conclusion, the dose of MLA used in this study was justified based on previous publications.

2.5.3. MLA has no effect on the reinforcing effects of heroin

The specificity of MLA for blocking the reinstatement phase was confirmed by its lack of effect on the acquisition of heroin CPP, which was in accordance with previous research by this lab (Wright et al., 2018) demonstrating that MLA had no effect on the acquisition of morphine CPP. This suggests that MLA did not have an effect on the reinforcing properties of heroin, as the association of the context (drug-paired side) with the cue (heroin) was established despite MLA pre-treatment. Furthermore, MLA was shown to have no effect on the expression, maintenance and reconsolidation of morphine CPP in mice (Wright et al., 2018). Together, these data suggest that α7nAChRs play a selective role in reinstatement of opioid CPP, but have no effect either on opioid reward, or on the ability of the animal to acquire opioid-induced CPP. Thus, these data support a selective role of α7nAChRs in modulating the reinstatement stage in the CPP model of opiate

relapse; which highlights α7nAChRs as potentially interesting targets in the study of relapsing behaviour.

In studies examining acquisition of nicotine CPP, the data are somewhat equivocal. Walters et al. (2006a) showed that antagonism of α7nAChRs by MLA (5 and 10mg/kg, s.c.) had no effect of the acquisition of nicotine CPP, with similar effects seen in self-administration paradigms using MLA (Grottick et al., 2000) or in α7nAChR knockout animals (Pons et al., 2008). In contrast, Harenza et al (2014) showed increased sensitivity for nicotine CPP acquisition in NAc α7nAChR knockout animals, indicative of enhanced nicotine-induced reward, whereas mice expressing a gain of function mutation of α7nAChR exhibited decreased sensitivity for nicotine CPP acquisition (Harenza et al., 2014). In the same study, however, MLA had no effect on acquisition of nicotine-induced CPP. Consistent with the idea that activation of α7nAChRs might decrease sensitivity to nicotine-induced reward is the study by Jackson et al (2017) who showed that pre-treatment of rats with the α7nAChR agonist, PNU282987, inhibited acquisition of nicotine-induced CPP. Opposite findings, however, were seen in the intravenous self-administration paradigm, where Markou and Paterson (2001) showed that MLA significantly reduced acquisition of intravenous nicotine self-administration. Overall, there is conflicting evidence as to whether α7nAChRs play a significant role in nicotine reward and associated learning paradigms, however it is possible that the effects of nicotine are blocked by either agonists or antagonists due to competitive nature (to a certain extent) for the same receptors or this effect is mediated by α7nAChR in other brain regions, such as the NAc (Harenza et al., 2014).

The evidence presented in this thesis for a selective MLA block of reinstatement but not acquisition is consistent with the literature on α7nAChRs in opioid-induced CPP.

2.5.4. Effect of MLA on decreased locomotion

Our lab previously observed an increase in locomotion in mice during the acquisition of morphine CPP and during morphine-primed reinstatement of CPP, which suggested a stimulant effect of morphine (Wright, 2016). This effect during reinstatement, but not acquisition was blocked by MLA pre-treatment. This stimulant effect of morphine and inhibition by MLA was also reported in reinstatement by Feng et al. (2011). Interestingly, during the present study, the opposite behaviour was noted. During acquisition, there was a slight (but not significant) decrease in locomotion (which MLA had no effect on, data not shown) and a significant decrease in locomotion during extinction, compared to habituation. Heroin priming significantly reduced locomotion during reinstatement and this effect was blocked by MLA. Heroin is a known drug of abuse with locomotor stimulant effects, like morphine (Andersen et al., 2009, Steidl et al., 2017), though there is a difference between studying the acute effect of heroin in a study on locomotion; and the present study which measures locomotor activity in a specific behavioural task. Tzschentke et al. (2006) noted a biphasic effect of heroin in CPP, where lower doses of heroin (0.05-0.25 mg/kg) had a locomotor stimulant effect and higher doses (1 and 3.16 mg/kg) produced locomotor reduction in the drug-paired compartment; which agrees with our findings.

As the post-conditioning test was conducted in a drug-naïve state, it is difficult to compare locomotion during reinstatement to post-conditioning (locomotion was not recorded during conditioning sessions). As heroin priming is presumed to involve the reactivation of the reward-associated memory (Bossert et al., 2013), it is possible that recall is strong enough to drive the rats to move over to the drug-paired side and remain there until the end of testing. The reversal of the decrease in locomotion by MLA could potentially be explained by the disruption of the re-activated memory, therefore the rats spent more time moving between the CPP chambers again as the association was lost. This theory was tested by analysing the number of cross-overs between CPP compartments (Figure 2.11). The heroin reinstatement group showed a significant number of transitions between CPP compartments, suggesting a reduction in exploration of the apparatus. This could be interpreted as the recall of the drug-paired association of the compartment was robust; therefore animals sought the drug-paired chamber and remained there without further exploration. Interestingly, there was no significant difference in the number of transitions between compartments in the MLA group, suggesting MLA potentially disrupted this drug-associated memory and a return to exploratory behaviour. There was however also no difference in the number of transitions compared to the heroin reinstatement group; therefore it cannot be determined whether MLA blocked this reduction in zone transitions. Due to the wide variability, additional subjects are needed to increase the power of the study.

2.5.5. Methodological considerations

2.5.5.1. Dose and route of heroin administration

A meta-analysis of opioid drugs in CPP experiments from 1979-1992 assessed the importance of various methodological factors in establishing drug-induced CPP with opioid drugs (morphine and heroin) and psychostimulants (cocaine and amphetamine) in rats (Bardo et al., 1995). Following their criteria, this meta-analysis found 208 groups of rats that had undergone CPP with the drugs mentioned above, and of these, only 39 were using heroin. For comparison, 76 were using morphine, demonstrating the scarcity of heroin CPP data in the literature. This comprehensive study showed there was a high correlation of heroin dose with the magnitude of CPP (Bardo et al., 1995). Doses of heroin ≥1 mg/kg produced the strongest CPP, and of the 8 studies in this category, 5 used the dose of 1mg/kg (Bardo et al., 1995). More recently, other studies have also shown that 1mg/kg heroin produces CPP in rats (Leri and Rizos, 2005, Tzschentke et al., 2006, Galaj et al., 2015, Galaj et al., 2016). This was therefore the dose of heroin chosen to condition the rats in this study. This meta-analysis covered publications between 1976 and 1992, therefore a more recent search showed only a few more studies using heroin CPP, which agreed with the previous meta-analysis that the dose of 1mg/kg produced CPP in rats (for review, see Tzschentke (2007)).

The same dose of heroin (1mg/kg) was also effective at producing reinstatement, in agreement with previous reports (Lu et al., 2002, Leri and Rizos, 2005). Although lower priming doses lower than the conditioning dose have been shown to be effective in reinstating CPP (to model an addict taking a small amount of

drug during abstinence, Do Couto et al. (2003)), there is no literature evidence for the effectiveness of a lower dose of heroin producing reinstatement of CPP.

2.5.5.2. CPP parameters

Other variables assessed by the meta-analysis were rat strain, sex, housing condition (grouped or individual housing), number of CPP compartments, route of administration of drugs, conditioning session number and duration and randomisation method (counterbalanced vs biased). They found that sex and rat strain had no effect on CPP. There was a strong effect of group housing and the presence of 3 CPP compartments (drug paired, unpaired and neutral). The majority of the studies analysed used the subcutaneous route of administration, however it was found that the intraperitoneal route may have a greater effect on (though only 6 cohorts used this route versus 33 subcutaneously). Due to the larger number of studies using subcutaneous administration, this was the route selected for the present study. Drug conditioning duration had a significant effect on CPP, whereas the number of conditioning sessions surprisingly had no effect. The CPP procedural parameters in this study were previously optimised by Wright et al. (2018), therefore these were not changed, but they were consistent with the strongest effects on heroin CPP shown by the meta-analysis (Bardo et al., 1995). The best CPP outcome for the randomisation method was the counterbalanced method; therefore this was used in the present study.

2.5.5.3. Clinical relevance of CPP

It is important to emphasise that CPP is not directly a model of addiction as rats receive a total of 2 heroin injections during the conditioning phase in this study. This is not enough to get the animals addicted, but it is more a correlate of the drug-paired associations which are formed during drug taking. These associations then become cues for drug-taking and can trigger cravings and push recovering addicts to seek drugs (Wikler, 1973, O'Brien et al., 1992). CPP is a conditioned behaviour where a contextual stimulus is paired with a primary reinforcer and acquires secondary appetitive properties. The test for CPP in a drug-free state enables an investigation of the salience of the drug-associated context. There were also no noted physical signs of heroin withdrawal (based on observations during testing) such as wet dog shakes, increased bowel movements, jumping, increased body temperature etc. (Pinelli et al., 1997) observed during the saline extinction stage, reinforcing the notion that CPP is not a direct model of addiction, but a correlate of reward-based learning.

CPP therefore provides a model for studying drug-seeking behaviour in response to a cue. A critique of this could be that the post-conditioning test is performed in a drug-naïve state therefore is not representative enough of human addiction, but this can be investigated further by using a model of drug-seeking where animals have volitional control of drug consumption, such as intravenous self-administration (IVSA). In the IVSA model, animals are in direct control of drug intake by pressing the correct lever and many different aspects of the motivation to seek a reward can be examined. This will be studied in Chapter 3.

2.5.6. Role of AMPA receptors in heroin reward-based learning

Previously, in our lab, autoradiographical quantification of total [^{3}H]-AMPA binding showed a 17% increase in binding in the ventral hippocampus only after morphine-primed reinstatement of CPP, and this was blocked by MLA (Wright et al., 2018). The aim of this study was to validate a method to quantify synaptic AMPA receptor expression at a subunit-specific level. Confocal and Li-Cor visualisation were completed side by side to investigate whether both of these methods would reveal similar patterns of expression.

Li-Cor visualisation was investigated because an entire brain slice could be scanned and protein expression levels could be quantified by immunohistochemistry in an entire brain region. The methodology is still relatively novel and unused. The methodology was based on a study by Eaton et al. (2016) who argued that image capture and analysis yielded comparable quantification to Western blot and confocal microscopy (see Appendix B for more detail). The advantages of using infrared scanning technology are that it enables area-based measurements in whole brain slices. Moreover, the time-cost benefits of whole-section image acquisition on an infrared system are considerable compared to confocal acquisition. A single brain slice could be scanned in approximately 5 minutes, whereas confocal capture for one brain slice would take approximately 90 minutes, incurring greater costs for booking the confocal microscope. Since the publication of the study (Eaton et al., 2016), only 2 other publications have cited using this methodology in brain slices,(Pellegrino et al., 2016, Chabrat et al., 2017) suggesting there could be limitations to this technique for protein quantification.

Confocal visualisation was selected as an adjunct to Li-Cor quantification as it could produce high resolution images and quantify AMPA receptor subunits at the

synapse. It was hypothesised that this could be a more sensitive method to detect smaller changes in protein expression. The signal intensity of secondary antibodies depends on a variety of factors such as the thickness of the slice, the depth of focus, the laser power settings and the image capture settings (such as gain and offset, Dunn et al. (2011)). Where possible, laser power and image acquisition settings were kept the same between slices, but other factors such as the depth of focus could still interfere with the signal intensity. To circumvent this, the colocalisation ratio of the AMPA receptor subunit with a control protein, PSD95 was used to normalise measurements between slices (see Appendix B for further detail). This was based on a publication by Zhang et al. (2014) who demonstrated a biphasic effect of glycine to induce long-term potentiation (LTP) or long-term depression (LTD) in rat hippocampal slices by the incubation in either a low (0.6mM) or high (1.5mM) concentration of glycine, respectively. They then used confocal microscopy to quantify the colocalisation of NMDA receptor subunits with PSD-95 in the dendritic region of the CA1. They found a significant 30% increase in GluN1 expression in this region following LTP induction by the low concentration of glycine, and a significant 40% decrease in GluN1 expression during LTD induction by the high concentration of glycine. They also found comparable changes in GluN1 expression by Western blot, demonstrating that the colocalisation of proteins could be used as a method of quantifying proteins consistently between brain slices. In this chapter, the measurement of colocalisation ratios failed to detect any effect of heroin priming or drug treatment on GluA1 or phosphorylated GluA1 expression, compared to saline control.

The hippocampus is a major target of cholinergic projections from the basal forebrain. α7nAChRs are highly expressed in the hippocampus where they

modulate glutamate-mediated synaptic plasticity (Cheng and Yakel, 2015). Enhancement of synaptic transmission is correlated with an increase in the number of synaptic AMPA receptors (Kessels and Malinow, 2009). One of the ways this is manifested is by the phosphorylation of GluA1 subunits at serine 845, which correlates with the delivery AMPA receptors to extrasynaptic sites, which primes them for LTP (Soderling and Derkach, 2000, Oh et al., 2006). Due to the increase in total [^{3}H]-AMPA binding observed previously in our lab (Wright et al., 2018) in morphine-primed rat brains, we hypothesised that heroin-primed reinstatement would be associated with an increase in the phosphorylation of GluA1 subunits, and potentially an increase in GluA1-containing AMPA receptors in the synapse. As the findings from the present study are equivocal, they neither agree nor disagree with previous findings. There are methodological issues which need to be addressed.

2.5.7. Methodological considerations

One major reason for the lack of detection of AMPA subunit expression changes using either method is the wide variability in the results. It was noted that day-to-day the measured signals varied greatly, suggesting a lack of consistency in the detection of signals. In the Li-Cor visualisation, brain slices from all treatment groups were scanned in parallel. With confocal visualisation, this was not possible, due to the time taken to acquire images; therefore this could contribute to the variability observed. As both methods of visualisation showed different trends in expression of these AMPA receptor subunits (as markers of LTP), it is difficult to determine for certain which method was more representative of the physiological changes in AMPA receptor expression occurring during reinstatement.

Billa et al. (2009) and Portugal et al. (2014) quantified synaptic AMPA or NMDA receptor expression (respectively) by isolating postsynaptic fractions using subcellular fractionation, and then quantified protein expression by Western blot. This group was able to quantify their receptors of interest at a subunit-specific level and reported changes in expression by an effect of drug treatment. I attempted to optimise this methodology for the purpose of this study, but was unable to replicate their findings (see Appendix A). Portugal et al. (2014) pooled mouse brain tissue to increase protein loading and detected changes in the pooled samples. For this study, I wanted to be able to detect changes at an individual level. Billa et al. (2009), however, were able to detect changes in AMPA subunit expression in individual rat brains, but due to time constraints and tissue limitations, I was unable to pursue subcellular fractionation further.

Interestingly, Billa et al. (2009) found that the extinction of morphine CPP in mice is associated with an increase in phosphorylation of GluA1 at serine 845. It could be that the lack of change in phosphorylated GluA1 is explained by the phosphorylation of serine 845 being a key element of extinction rather than reinstatement. Extinction is however also a form of learning, as the association of the CPP compartment is re-written to no longer associate with the euphoric effect of the drug (Bouton, 2002); therefore it could be argued that the reinstatement of CPP could be triggering similar mechanisms of LTP as extinction. This hypothesis however conflicts with evidence from Portugal et al. (2014) who showed a disruption of LTP in the hippocampus during extinction of morphine CPP in mice, but a robust enhancement of LTP during reinstatement. Further work is needed to differentiate the molecular mechanisms underlying the motivational behaviour behind the different stages of CPP, as this is still unclear.

2.5.8. Contribution of other AMPAR subunits?

There could also be changes in the expression of other AMPA receptor subunits, such as GluA2, which is also important in the modulation of synaptic plasticity (Isaac et al., 2007). This subunit is however more involved in long-term synaptic plasticity and the stabilisation of the synapse by the replacement of GluA1 homomeric AMPA receptors with GluA2-containing AMPA receptors, which occurs over a period of hours (Isaac et al., 2007). We attempted to quantify these subunits as well, but due to technical issues with antibodies, there were insufficient data to be shown. It would be interesting in the future to quantify GluA2 subunits alongside GluA1, or to examine time-dependent changes in AMPA receptor expression by delaying the time of rat sacrifice after reinstatement

2.5.9. Implication of the role of α7nAChR antagonism in opioid addiction

Learning and memory are key elements of opioid addiction (Hyman et al., 2006, Isokawa, 2012) and in animal models, it has been shown that glutamatergic synaptic plasticity is a major substrate of the reward-associated learning paradigm that is CPP (Van den Oever et al., 2010, Portugal et al., 2014, van Huijstee and Mansvelder, 2014). There have been previous reports of nicotinic modulation of memory recall but these studies have been limited to stress- and emotion-related memory rather than to reward-based learning (for review, see Blake et al. (2014)).

Bitner et al. (2007) showed that selective agonism of α7nAChRs increased cognitive performance in monkeys in a model of working memory, increased rat

social recognition as a model of short-term recognition memory and increased performance in a mouse model of long-term memory consolidation. Furthermore, the selective agonism of α7 nAChRs reversed sensory gating deficits induced by MLA in mice (Bitner et al., 2007). At a molecular level, α7 nAChR agonism increased the phosphorylation of downstream molecules (ERK1/2 and CREB) involved in memory consolidation processes involving calcium in the mouse hippocampus (dorsal or ventral was not specified, however images suggest dorsal). The α7nAChR subtype is therefore of particular interest as it is also calcium-permeable, suggesting a potential intervention in the phosphorylation of the downstream signalling molecules involved in memory processes (see Chapter 1, section 1.4).

Evidence from our laboratory has shown that MLA attenuates evoked LTP in the mPFC in mouse brain slices, suggesting a role of endogenous ACh in regulating glutamatergic signalling (Udakis et al., 2016), in concordance with others (Gu et al., 2012, Cheng and Yakel, 2015). Sabec et al. (2018) recently demonstrated that α7nAChRs expressed in the PFC are critical for the encoding of associative recognition memory in rats. Furthermore, they also found that α7nAChRs gate bi-directional plasticity at hippocampal-prefrontal synapses. These findings, demonstrate an essential role of α7nAChRs in regulating synaptic plasticity in learning and memory processes in the PFC and hippocampus. As synaptic plasticity underlies memory processes, it can be reasoned that antagonism of α7nAChRs will affect memory processes in these brain regions.

In order to determine the precise brain region mediating the inhibitory effects of MLA, bilateral intracerebroventricular (i.c.v.) infusions into different rat brain regions (PFC, ventral HPC, dorsal HPC) were performed before morphine-primed

reinstatement (Wright et al., 2018). Only infusions of MLA into the ventral HPC significantly inhibited morphine-primed CPP. The vHPC is a brain region known to be the site of memory processing and the modulation of reward-related memories (Isokawa, 2012, Namba et al., 2018). These data fit in with the theory that the antagonism of α7nAChRs inhibits reward-related memory, and more specifically, the re-activation of those memories in reinstatement.

2.6. Conclusions

The behavioural data shown here demonstrate that:

1) α7nAChRs do not mediate the primary reinforcing effects of heroin
2) α7nAChRs play a selective role in the reinstatement phase of heroin CPP

Although CPP is a robust method to study the drug-paired associations which act as triggers for relapse, this is a passive method of motivational memory. To explore further the role of alpha7 receptors in opiate reinstatement, MLA was tested in a more active model of volitional drug consumption: the reinstatement of intravenous self-administration of heroin.

CHAPTER 3 THE ROLE OF α7 NACHRS IN HEROIN INTRAVENOUS SELF-ADMINISTRATION

3.1. Introduction

3.1.1. Intravenous self-administration

As discussed in the introduction (Chapter 1, section 1.3.2), intravenous self-administration (IVSA) is an operant behavioural paradigm which is often used as a correlate of the complex aspects of motivational behaviour towards a drug reward in relapse in humans.

In this study, animals were trained to self-administer heroin intravenously by pressing the active lever on an FR5 schedule of reinforcement. Subsequently, the behaviour was extinguished by the heroin reward being replaced with saline in the pump syringes. Three different aspects of motivation to seek the drug reward were investigated: the relative reinforcing effect of heroin, the re-acquisition of heroin IVSA, and the reinstatement of IVSA. The effect of antagonising α7nAChRs by MLA was investigated in each of these motivational states. Using a combination of tone and light cues with drug priming during reinstatement has been shown to have an additive effect on reinstatement compared to either priming method alone (Shelton and Beardsley, 2008, Fattore et al., 2010, Keogh et al., 2017), therefore for the purpose of this study, the combination of drug and cue-priming was used to induce the reinstatement of IVSA.

The heroin IVSA was performed at RenaSci Ltd (Nottingham, UK). The self-administration protocol was based on previous studies performed by RenaSci (Heal et al., 2013, Keogh et al., 2017), with doses of heroin widely reported to be effective in producing self-administration in rats (Stewart, 1983, Shalev et al., 2002). Furthermore, the doses used in this study were based on dose-response studies at RenaSci (data not shown). As mentioned in the introduction (Sections

1.3.4.1 and 1.3.4.2), drug-primed and cue-primed reinstatement behaviours are governed by distinct, yet overlapping neurobiological substrates (Rogers et al., 2008, Namba et al., 2018). In most studies, the two triggers of reinstatement are used separately in experiments. Studies have found that while cues **or** stress significantly prime the reinstatement of drug-seeking alone, the combination of both triggers has an additive effect on reinstatement of ethanol seeking (Liu and Weiss, 2002), cocaine seeking (Buffalari and See, 2009, Feltenstein et al., 2011) and heroin seeking (Banna et al., 2010). The combination of drug **and** cue-priming has however been rarely investigated. Shelton and Beardsley (2008) have found that the combination of cues and methamphetamine-priming has an additive effect on the reinstatement of drug seeking in rats. This effect has been further demonstrated with cannabinoids (Fattore et al., 2010) and more recently, cocaine (Keogh et al., 2017); though this has yet to be shown with heroin.

The reinstatement of drug seeking can be triggered by non-contingent drug priming, the presentation of conditioned cues and stress in animals previously trained to self-administer drugs. Chapter 1 (Figure 1.9, section 1.3.4) demonstrated the distinct and overlapping pathways involved in drug- and cue-primed reinstatement of drug seeking, and this chapter focusses on the evidence of α7nAChR modulation of this drug-seeking behaviour.

3.1.2. The role of α7nAChR in reinstatement of self-administration

There are few reports of the role of α7nAChRs in the reinstatement of self-administration of drugs of abuse, and none currently that examine their role in heroin relapse.

As discussed in the introduction (section 1.4.5), there is a distinct differential role of nAChR subtypes in nicotine reinforcement and the cue-induced reinstatement of nicotine IVSA. Studies have demonstrated that while β2-containing nAChR were essential during the acquisition stage of nicotine self-administration, α7nAChRs were important in cue-primed reinstatement of nicotine seeking (Liu et al., 2007, Cahir et al., 2011, Liu, 2014). Furthermore, transgenic α7nAChR knockout mice were still capable of acquiring nicotine IVSA, whereas α4 and β2 subunit knockout mice were not (Pons et al., 2008). It was however shown in a contradicting study that there was no difference in nicotine self-administration or CPP compared to wild-type in α4 knockout mice (Cahir et al., 2011, Madsen et al., 2015). Nicotine self-administration was rescued in the α4 and β2 subunit knockout mice by their re-expression in the VTA, but not in the SN (Pons et al., 2008). In addition, MLA pre-treatment had no effect on the acquisition of nicotine CPP (Pons et al., 2008), and this was further demonstrated in α7nAChR knockout mice (Walters et al., 2006b).

This distinction of the specificity of α7nAChRs in self-administration behaviour was further demonstrated in squirrel monkeys (Secci et al., 2017). Secci et al. (2017) found that increasing levels of kynurenic acid, a proposed negative allosteric modulator of α7nAChRs, inhibited cue-primed reinstatement of nicotine IVSA in rats and inhibited cue-primed and drug-primed reinstatement of nicotine and cocaine IVSA in squirrel monkeys. They also demonstrated this effect on the drug-primed and cue-primed reinstatement of THC self-administration in monkeys in another study (Justinova et al., 2013). They demonstrated the inhibition of reinstatement was reversed by the α7nAChR PAMs PNU120596 (Secci et al., 2017) and galantamine (Justinova et al., 2013). Increasing brain kynurenic acid

levels however also decreased the acquisition of nicotine self-administration in monkeys, which was inhibited by PNU120596, and this effect was not shown in rats (Secci et al., 2017), suggesting either that there are species differences involved, or that some of the effects of kynurenic acid were not mediated by α7nAChRs; but rather by competitive inhibition of the glycine co-agonist site of the NMDA receptor, or by antagonising AMPA or kainate receptors (which kynurenic acid binds to at higher concentrations, Bertolino et al. (1989), Prescott et al. (2006), Albuquerque and Schwarcz (2013)). Furthermore, kynurenic acid levels were found to be increased in the VTA and NAc shell in rats (Justinova et al., 2013) and this reduced the ability of nicotine to stimulate dopamine release in the NAc shell. This effect was however not reversed by PNU120596, suggesting this inhibition of dopamine release could be via the antagonism of glutamate receptors rather than α7nAChRs. Additionally, kynurenic acid inhibited the acquisition of nicotine but not cocaine IVSA, and this effect was rescued by the α7nAChR PAM in monkeys. This conflicts with the previous findings by Liu (2014), who showed no effect of MLA on the acquisition of nicotine IVSA. These data are intriguing and could suggest a potential effect mediated by α7nAChRs in these addiction-related behaviours; however the non-selectivity of kynurenic acid for glutamate receptors, which mediate a variety of cognitive functions including reward-based learning, casts some doubt over the role of α7nAChRs in mediating these results. Further work with more selective compounds is needed to enhance these findings.

The evidence discussed above and the findings in Chapter 2 have identified an intriguing role of α7nAChRs in selectively being involved in mediating relapse-like behaviour. This has yet to be shown in the reinstatement of self-administration with opioid drugs, which this study will address.

3.2. Aims of this chapter

The aims of this chapter were to determine the role of α7nAChRs in different motivational states in a rat model of heroin intravenous self-administration. Based on publications and the findings in Chapter 2, we hypothesised that:

1) MLA would have no effect on the acquisition of lever pressing

This was investigated by observing the effects of MLA pre-treatment on the lever pressing behaviour for a food reward.

2) MLA would have no effect on the primary reinforcing effects of heroin

This was investigated by observing the effects of MLA pre-treatment on the re-acquisition of heroin IVSA (heroin infusions) and on the relative reinforcement of heroin by analysing the breakpoints in a progressive ratio session.

3) MLA would inhibit the reinstatement of drug seeking

This was investigated by observing the effects of MLA pre-treatment on the combined drug- and cue-primed reinstatement.

3.3. Materials and Methods

3.3.1. Drugs

All drugs were made up in sterile sodium chloride solution (0.9% w/v, Baxter, UK) and filter sterilised using 0.2µm filters into sterile tubes or glass bottles. Heroin (MacFarlan Smith, UK) stock solutions (1 mg/mL) were prepared once weekly, the pH adjusted to pH 6.6-7.4, and stored at 4℃ when not in use. Heroin dosing solutions (0.05 and 0.015 mg/kg/infusion) were prepared from stock when required and the pH adjusted to pH 6.6-7.4. The training doses were selected due to previous studies showing they were effective in inducing heroin self-administration in rats (Shalev et al., 2002). Drug syringes were emptied at the end of each testing day and stored at 4℃ overnight. Each day, they were re-filled with fresh heroin or saline solution each morning of testing.

MLA (Abcam) dosing solutions (4mg/mL) were prepared in sterile saline, filter sterilised, aliquoted into 2 mL cryovials and stored at -20℃ for up to one month, according to the manufacturer's recommendations. The dosing volume for subcutaneous injection was 1mL/kg and MLA was dosed at 4mg/kg, s.c. 20 minutes prior to each of the reinstatement sessions. The control group was dosed with 1mL/kg sterile saline prior to reinstatement.

3.3.2. Animals

All experiments were performed in accordance with Home Office project licence held under 'ASPA' 1986 and approved by a local ethical review panel. 26 male Sprague-Dawley rats (200-225g, Charles River) were housed in the same room as the testing boxes. On arrival, they were weighed on arrival and singly-housed in

large polypropylene cages containing rodent bedding and chew sticks. Cages were cleaned once a week and bottles twice a week to maintain hygiene. Experiments were carried out in the light phase (12-hour light/dark cycle, 07:00 on/19:00 off) in a temperature-controlled (21℃ ± 4℃) and humidity-controlled room (55% ± 20%).

3.3.3. Housing and diet

Rats were given free access to food in food hoppers on arrival but were later mildly food restricted after acclimatisation. The day after their arrival, rats were weighed to monitor for overnight weight loss due to the stress of travelling and none was noted. They were handled and weighed each day for one week during the acclimatisation period (except on the weekend) to habituate them to the experimenters. Post-surgery, the cages were modified to prevent access to the underneath of the food hopper, to prevent causing damage to the in-dwelling catheter ports implanted in the back of the rats' shoulders.

Rats were fed a standard rodent diet of Teklad 2018 (Envigo, UK). Due to the modified cages, food was placed directly onto the cage floor, but water was accessed normally via water bottles. During the acclimatisation period, they were also fed approximately 3 food training pellets (45 mg dustless precision pellets; F0021-B, Bilaney Consultants Ltd.) with their Teklad 2018 diet each day, so that the rats recognised the pellets as food when they start lever-press training for food rewards. After the acclimatisation period, food was restricted to 10g/day over 5 days. After this time, daily food intake was restricted to 90% of normal levels (normal levels were initially calculated as the mean daily food intake during the

acclimatisation period). Thereafter, body weights were monitored daily and the amount of food was adjusted to maintain age-appropriate growth. This regime was maintained throughout the remainder of the study, except for a 24 hour period pre-surgery and a 48 hour recovery period from surgery, during which time animals had free access to food. Approximately 48 hours post-surgery, daily food intake was again restricted to 90% of normal levels.

3.3.4. Bodyweights

Each rat was weighed daily from their date of arrival until the end of the experiment. The bodyweights were recorded and the food weight was adjusted according to bodyweight gain or loss (see below for further details). The figure below shows the mean bodyweights of all rats during the experiment.

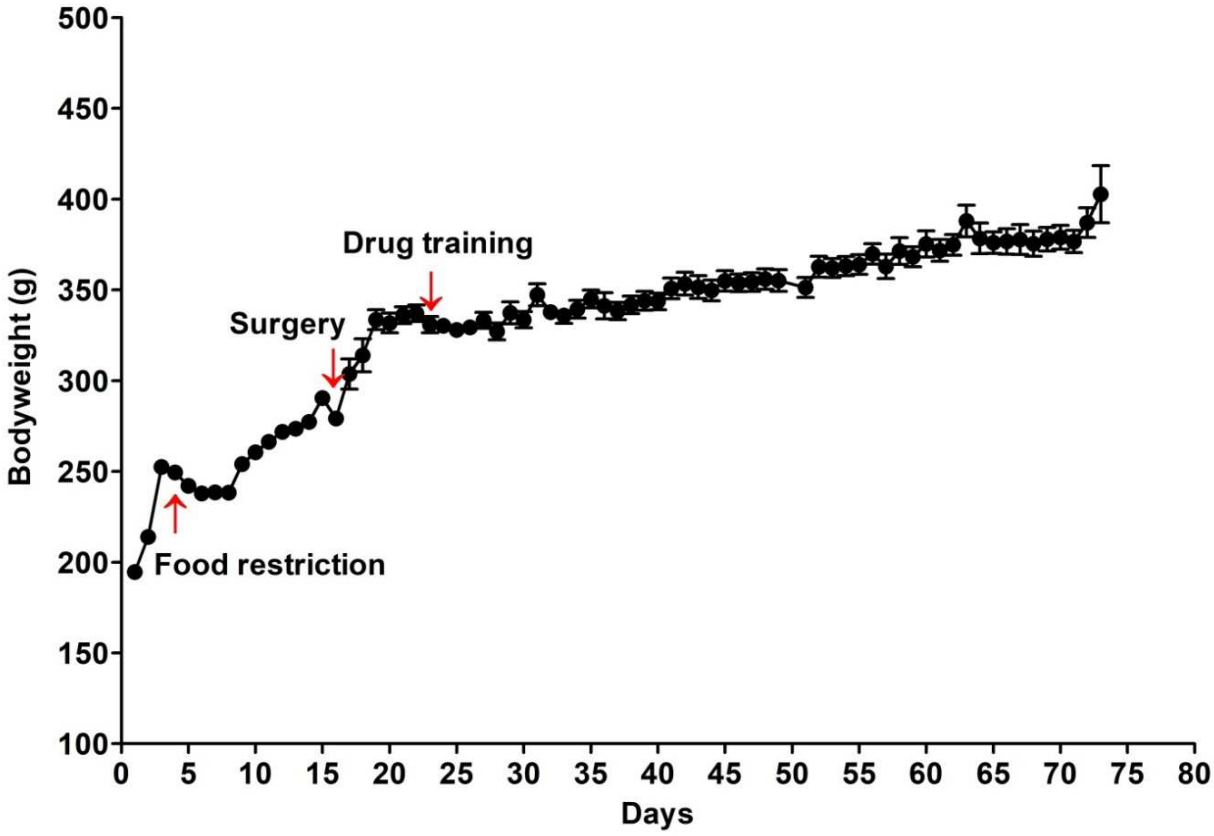


Figure 3.1: Bodyweights of rats for the duration of the heroin intravenous self-administration experiment. Data points are mean bodyweights of the rats used in the analysis of this study, after experimental exclusions. Red arrows represent time points of food restriction (day 4), surgery (from day 16) and drug training (from day 23). Surgeries were staggered and performed during the week starting day 16, therefore drug training was also staggered and began day 23. Data are mean±SEM, n=14.

3.3.5. IVSA apparatus

Eight operant chambers (MED Associates, Inc., St. Albans, Vermont, USA) located within sound-attenuating, ventilated cubicles (Model ENV-018MD; MED Associated, Inc.) were used for the duration of the experiment. The apparatus consisted of a chamber with Perspex walls and one stainless steel wall with two response levers, either side of a 5x5 cm opening located equidistant between the

levers available for food pellet delivery from a food hopper. The chamber was fitted with a house light, a 2.5 cm translucent stimulus light located above each lever and an audible tone generator. Data were collected and stored by a microprocessor and associated interface (MED Associates, Inc.).

Each sound attenuating cubicle was equipped with an infra-red camera (Model 170IR, RF concepts) from which images were relayed to a digital video recorder (Model RF2421, RF Concepts) and displayed on a computer monitor. The cameras allowed the operator to monitor animal activity inside the chambers, thus decreasing the time taken for acquisition of lever-pressing (by manually dispensing food pellets to aid learning) and preventing chewing of the access port tethers when connected.

Figure 3.2: Images of operant boxes (**A** and **B**) which are housed in sound attenuating cubicles **(C)**. Panel **D** shows a rat attached to port tethers for self-administration. Images from RenaSci.

3.3.6. Acclimatisation

During the 6 days of acclimatisation, rats were handled and weighed daily and food intake was recorded. In addition to their food, rats were given 3 food training pellets each to introduce them to the pellets used as a food reward during training sessions.

3.3.7. IVSA procedures

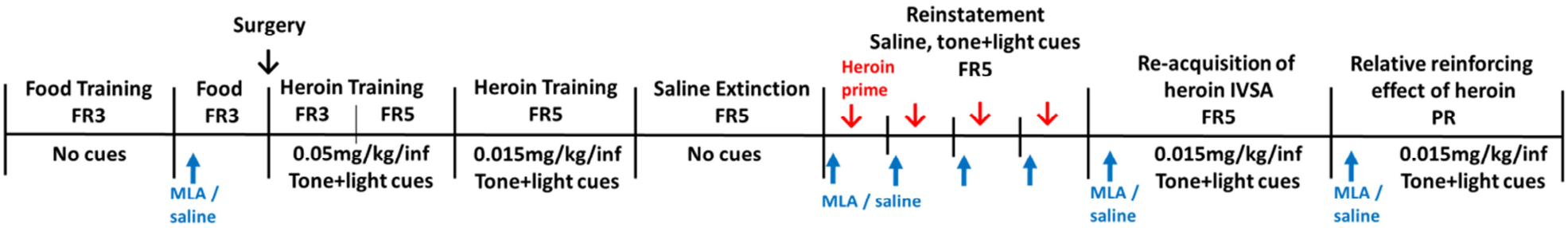


Figure 3.3: Schematic of the experimental procedures for the duration of heroin intravenous self-administration. FR: fixed ratio of reinforcement, PR: progressive ratio. Upward blue arrows represent MLA (4 mg/kg, s.c.) or saline (1 mL/kg, s.c.) pre-treatment 20 minutes prior to experiments. The downward black arrow represents rats undergoing surgery for intravenous catheterisation. Downward red arrows represent heroin priming (0.015mg, i.v.) immediately prior to being placed in operant boxes. During drug-and cue-primed reinstatement, rats were pre-treated with MLA (or saline, blue arrows) 20 minutes prior to heroin priming (red arrows) over 4 consecutive days.

Study Phase	Contingent cues (light + tone)	Contingent heroin infusion	Contingent saline infusion	Heroin priming injection immediately prior to the session	Non-contingent cues (light + tone) to start the sessions	Non-contingent heroin infusions to start the sessions
Food training	No	No	No	No	No	No
Heroin acquisition (light + tone cues)	Yes	Yes	No	No	Yes	Yes
Saline extinction	No	No	Yes	No	No	No
Heroin i.v. primed + cue induced reinstatement	Yes	No	Yes	Yes	Yes	No
Re-acquisition of heroin self-administration	Yes	Yes	No	No	Yes	Yes
Relative reinforcement of heroin (PR)	Yes	Yes	No	No	Yes	Yes

Table 3.1: Summary of testing conditions for each stage of the heroin IVSA study. The house light was illuminated in the boxes at all times. It did not go out during the 30 second time-out after each pump activation.

3.3.7.1. Food training

During food training, animals were food restricted to 10 g of standard rat chow per day to encourage rats to seek food rewards. Rats were weighed and placed in the apparatus to learn to press levers with a food reward. Light and sound cues were not presented at this time (see Figure 3.3 and Table 3.1 for details). Initially, rats were placed on a fixed ratio 1 (FR1) schedule, where 1 lever press dispensed one 45 mg pellet of food and both levers were active. The maximum duration of the food training session was 1 hour with a cut-off of 50 food pellets if this was reached first. Once rats self-administered over 45 pellets on FR1, they were progressed either onto a final FR3 schedule of reinforcement where 3 lever presses were required to dispense 1 food pellet and only the designated active lever (left lever) would dispense a pellet. If the rats showed a preference for the

inactive lever, they were progressed onto the FR2 schedule and thenFR3 to aid learning. The number of food training sessions was kept to a minimum to establish a robust lever pressing response; responding was deemed stabilised on the FR3 schedule for all rats when animals took over 45 pellets per session for 4 consecutive days.

At the end of each session, a maximum of 3 rats were placed in an enrichment area to socialise before being returned to their home cages. After all rats had completed the session, boxes were cleaned for the following day. All operant chambers were cleaned with 10% ethanol and the collection trays underneath the boxes were cleaned with Virkon at the end of the day.

3.3.7.2. Effect of MLA on lever pressing

When rats were considered trained for food lever-pressing (over 45 pellets per session for 4 consecutive days), 12 rats were randomly allocated to either saline or MLA pre-treatment group in a pilot study to assess the effect of MLA on lever pressing (for food reward, see Figure 3.3). 20 minutes prior to the lever pressing session, they received either saline (1mL/kg, s.c) or MLA (4 mg/kg, s.c.). They were then placed in the operant chambers to lever press for food pellets on an FR3 schedule for the maximum allotted time of 1 hour or until the maximum 50 pellets were dispensed. Abnormal behaviours such as piloerection, head weaving and ataxia were monitored for regularly and recorded until 2.5 hours post-injection. At the end of the experiment, rats were prepared for surgery as described in the following section.

3.3.7.3. Surgery

All surgical procedures were performed by RenaSci staff, due to time constraints and the complexity of the procedure. Surgery was staggered, so that a maximum of 6 rats underwent surgery per day. 24 hours before surgery, rats were given free access to food. On surgery day, rats were weighed and administered with the antibiotic Baytril (5mg/kg, s.c.) and the anti-inflammatory carprofen (80mg/kg, s.c.), then anaesthesia was induced with inhalable isoflurane and the relevant areas were shaved. Practising aseptic technique, the catheter (silicone tubing, RenaSci Ltd) was implanted into the right jugular vein, secured to the vessel then tunnelled subcutaneously from the site of insertion to the mid-scapular region where the access port was to exit. The access port was implanted such that the direction of the cannula and catheter pointed rostrally, and then sutured into place to prevent the port from twisting. The wound was closed with sutures and dressed with antiseptic spray. Catheters were filled with sterile fluid (heparinised saline, 30iU/mL) immediately post-surgery and after every experimental session to maintain catheter patency. Catheter patency was confirmed daily by drawing back and observing freely flowing blood in the catheter line. In 1 rat out of 26, blood was not drawn after surgery, so the rat was intravenously injected with a sedative (propofol, 1.625mg/kg, i.v.). As the rat failed to show immediate sedation upon injection of the propofol, the animal was terminated by a UK Home Office Schedule 1 procedure.

Immediately following surgery, the rats were placed in cages with modified lids under an infrared lamp and with the cage on top of an electric blanket; with free access to food and wet mash on the cage floor. Their recovery from the

anaesthetic was closely monitored and they were routinely checked until 1 hour after consciousness was regained.

Rats were allowed to recover from surgery for 48 hours during which time their catheters were not flushed. The sutures and general behaviour and aspect of the rats were checked. In the rare case of animals removing their sutures, the wounds were glued shut with tissue-appropriate superglue (GLUture, Abbott Laboratories). They were all administered Baytril (5mg/kg, s.c.) for 48 hours and weighed to note any post-surgery weight loss, and were given free access to food and water. Two rats were euthanised by Schedule 1 procedure to minimise suffering, due to them pulling out their sutures and opening their glued incisions multiple times.

48 hours post-surgery, rats were weighed, sutures were examined and home-cage behaviour was recorded. The cannula port metal screw-on lid and plastic cap were removed and sterilised in 70% ethanol. The antibiotic timentin (ticarcillin clavulanate, 80 mg/kg, i.v.) was administered via the catheter, which was then flushed with heparinised saline (30 iU/mL) to prevent blockages. This was repeated daily until the end of the study. The plastic cap and metal screw-on lid were then coated with the antibacterial Posatex and tightly shut. The plastic caps and metal ports were cleaned as such daily. Rats were returned to the 90% food restriction they were on prior to surgery.

3.3.7.4. Acquisition of heroin self-administration

After recovery from surgery for 6-7 days, rats were trained to self-administer heroin in daily two-hour sessions (Figure 3.3).

Prior to drug training, the operant chamber food hoppers were emptied of any residual food training pellets and cleaned with 10% ethanol to remove any food smells, to ensure the rats do not continue to be motivated by food when lever pressing. The Skinner boxes were set up with infusion pumps with syringes and tubing running from the syringes was fed through metal tethers and flushed with the ethanol then air daily prior to filling with drug or saline. Sessions were started by a non-contingent infusion of heroin and simultaneous presentation of a stimulus cue light and a 2.9 kHz tone cue set at 65 dB (approximately 5 dB over the background noise in the boxes). These two cues were given for a 5 second period contingent with heroin drug infusions during the training period following the correct number of lever presses, corresponding with the approximate duration of drug infusion. The infusion volume was 0.4 ml / kg.

Training was started on an FR3 schedule where heroin infusions were limited to a maximum of 20 per 2 hour test session. During sessions, the required number of presses on the active lever (the left lever) resulted in the delivery of a single i.v. heroin injection (0.05 mg/kg/injection heroin base) that was given as a 5 second infusion according to the bodyweight of the rat. Each infusion was accompanied by the presentation of the contingent light and tone cues. Each heroin infusion was followed by a 30 second time-out period during which time presses on the active lever would have no programmed consequences. The house light remained illuminated throughout the test sessions.

At the end of each session, the catheters were flushed with 1mL/kg timentin (80mg/kg) and with 0.2mL heparinised saline (30iU/mL) and the plastic and metal caps were coated in the antibacterial Posatex and replaced. Up to 3 animals (no

more to prevent any damage to catheter ports) were placed together in a socialisation pen for up to 15 minutes at the end of their session to allow some socialisation time and reduce stress. At the end of the day, the operant chambers were cleaned with 10% ethanol and the collection trays cleaned with Virkon.

Initially, rats were trained on 0.05 mg/kg heroin. When rats had learned lever pressing on FR3, the schedule was increased to an FR5 schedule. When lever pressing was stabilised, the heroin dose was reduced to 0.015 mg/kg on the FR5 schedule, a dose also shown to produce self-administration in rats (Shalev et al., 2002) with a maximum of 20 infusions per 2 hour session.

The acceptance criterion for positive reinforcement for heroin acquisition was defined as three consecutive sessions where the mean number of infusions was 12 or above. Once this criterion was met, animals were ready for saline extinction of responding. 8 rats of the remaining 23 did not reach the acceptance criteria for heroin self-administration acquisition and were euthanised by a Schedule 1 procedure.

3.3.7.5. Extinction

Rats that met the acquisition criterion above were transitioned into FR5 saline extinction in the absence of cues for 2 hour sessions with a maximum of 25 infusions (see Figure 3.3 and Table 3.1). Syringe pumps were filled with saline and the session was started immediately in the absence of the cues or a non-contingent infusion. On the FR5 schedule, rats received an infusion of saline with no cue presentation, with a 30 second time-out between infusions, for 2 hour sessions. The house light remained illuminated throughout the session. The room

was kept as quiet as possible as noise could interfere with extinction, and rats were kept in the operant chambers during the whole 2 hour period, regardless whether they reached the maximum number of infusions, to emphasise the lack of reward. At the end of the sessions, rats received 2-3 s.c injections of saline (1 mL/kg) to acclimatise them to subcutaneous injections before reinstatement.

Rats underwent a minimum of 5 sessions. The acceptance criterion for non-reinforcement with saline was defined as three consecutive sessions where the mean number of lever presses was ≤30% of the mean active lever presses during the last three heroin acquisition sessions. All of the remaining rats achieved the extinction criterion and proceeded onto reinstatement.

3.3.7.6. Heroin- and cue-primed reinstatement of drug seeking

Rats were subjected to heroin i.v. primed- and cue-induced reinstatement of responding on an FR5 schedule, for 4 consecutive days, with saline as contingent infusions (Figure 3.3). The 2 hour sessions were initiated by non-contingent presentation of the tone and light cues (Table 3.1). Animals were pseudo-randomly assigned to one of two treatment groups:

Group 1: Saline (1mL/kg, s.c.) + heroin i.v.-primed and cue-induced reinstatement

Group 2: MLA (4mg/kg, s.c.) + heroin i.v.-primed and cue-induced reinstatement

Saline or MLA was administered s.c. 20 minutes prior to the reinstatement sessions. The heroin priming dose (0.015 mg/kg) was administered by i.v. through the indwelling catheter immediately before the test session. During the reinstatement sessions, upon the correct number of lever presses (FR5), rats

received a saline infusion paired with the light and tone cues over 5 seconds. There was a 30 second time-out after each infusion, during which active lever presses had no programmed consequences. The house light remained illuminated for the duration of the session. The rats were given 4 reinstatement sessions over consecutive days.

This reinstatement test aimed to observe the effect of MLA pre-treatment on drug seeking behaviour; therefore data were presented as the number of active lever presses during each session.

3.3.7.7. Re-acquisition of heroin self-administration

After the rats completed reinstatement of drug seeking, they were re-extinguished on saline as described in section 3.3.7.5 for 2-3 days until the animals achieved the criterion for extinction of lever pressing. They were then progressed onto the re-acquisition of heroin self-administration test, where heroin (0.015 mg/kg/inf) was available in the syringe pumps on the FR5 schedule of reinforcement (Figure 3.3). The rats remained in their treatment groups and were pre-treated with either saline (1 mL/kg, s.c.) or MLA (4 mg/kg, s.c). 20 minutes prior to the reinstatement session. They were then placed in the Skinner boxes and the test session was initiated by a non-contingent infusion of heroin (0.015 mg/kg/inf), simultaneously presented with the tone and light cues for 5 seconds (Table 3.1). Each successful infusion was contingently presented with the tone and light cues, followed by a 30 second time-out period where active lever presses resulted in no programmed consequences. Rats underwent a maximum of 6 re-acquisition sessions and received saline or MLA before each session, until responding was stabilised and

they reached the heroin acquisition criteria (≥12 infusions for 3 consecutive sessions). The last 3 sessions were taken for analysis.

This test aimed to observe the effect of MLA pre-treatment on the re-acquisition of heroin IVSA; therefore data were presented as the number of infusions received during the session.

3.3.7.8. Relative reinforcement of heroin

Upon completion of the re-acquisition of heroin self-administration, rats were given a single progressive ratio (PR) session for heroin (0.015 mg/kg) in a four-hour session where the number of lever presses progressively increased for a single drug infusion (Figure 3.3). Table 3.2 shows the progressive ratio schedule and the escalating number of lever presses for a single heroin reward. Animals remained in their treatment groups and received saline (1 mL/kg, s.c.) or MLA (4 mg/kg, s.c.) 20 minutes prior to the beginning of the session, which was then initiated by non-contingent presentation of the tone and light cues. PR results were presented as the mean break-points for each group.

Presses	5	7	9	12	15	20	25	30	40	50	62	77	95	118	145	178	219	268	328
Rewards	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	19	20

Table 3.2: Number of lever presses required for subsequent rewards on progressive ratio schedule of reinforcement.

3.3.8. End of study

At the end of the study, all rats were terminated using a UK Home Office Schedule 1 method. The brains were not collected due to multiple experiments done on the same rats.

3.3.9. Statistical Analysis

All heroin intravenous self-administration data were presented as mean ± standard error of the mean (SEM). The total number of drug infusions, active and inactive lever presses per session in the drug training, extinction, reinstatement and relative reinforcement of heroin were recorded by Med Associates software.

The total number of active lever presses in the drug- and cue-primed reinstatement of drug seeking sessions was recorded and compared statistically with those obtained for the mean of the last 3 extinction sessions.

The total number of heroin infusions in the re-acquisition of heroin self-administration was recorded and compared statistically to the mean number of infusions over the last 3 extinction sessions.

All statistical analysis on the raw data was conducted in GraphPad Prism version 5.

In some cases (5 minute time bins, time to first lever press, inter-injection interval and breakpoint), the statistical analysis was performed by RenaSci's in-house statistician. The data was transformed by square root transformation, then back-

transformed to normalize the data. Once the transformation was completed, the statistical methods could assume normal distribution with equal variance. The 5 minute time bin data and time to first lever press were analysed by mixed linear model with treatment as a fixed factor and animal as a random factor. Comparisons within treatment groups and to saline extinction were by the multiple t test. PR breakpoints were analysed by unpaired t test.

3.4. Results

3.4.1. Effect of MLA on lever pressing

To determine if MLA had any effect on lever pressing, a pilot study was conducted on a small cohort (n=6 for both groups) before drug training. Once animals had reached a stable number of food rewards, they received a subcutaneous (s.c.) injection of either saline (1 mL/kg) or MLA (4 mg/kg) – the same dose shown to inhibit morphine-primed reinstatement of CPP in previous studies (Feng et al., 2011, Wright et al., 2018) and the heroin-primed reinstatement of CPP in Chapter 2 (Section 2.4.3.) – 20 minutes prior to the 2 hour food reward session on an FR5 schedule. The behaviour was noted and scored before, during (using cameras in the operant boxes) and after the food reward session at specific time-points (see Table 3.3 and 4). This would determine any behavioural effects of MLA and whether this could account for any future changes in responses in IVSA.

Group A							
Vehicle: Saline 1.0 mL/kg s.c.							
Behaviour/physical signs	**Time Point (min)**						
	5	**15**	**30**	**60**	**90**	**120**	**150**
Piloerection (mild)					1/6	4/6	
No observed change	6/6	6/6	6/6	6/6	5/6	2/6	6/6

Table 3.3: Observations of the effect of saline on rat normal behaviour. Scores are represented as the number of rats displaying the behaviour over the total sample size.

Group B							
MLA 4 mg/kg s.c.							
Behaviour/physical signs	**Time Point (min)**						
	5	**15**	**30**	**60**	**90**	**120**	**150**
Piloerection (mild)			2/6	1/6	1/6	1/6	
Head weaving (mild)		1/6	1/6	1/6	1/6		
Subdued (mild)		1/6					
Body twitches (mild)						1/6	
No observed change	6/6	4/6	4/6	4/6	4/6	4/6	6/6

Table 3.4: Observations of the effect of MLA on rat normal behaviour at specific timepoints. Scores are represented as the number of rats displaying the behaviour over the total sample size.

All of the behaviours noted post-MLA treatment were mild in nature. Some mild piloerection was noted during this test (Table 3.4), but as it was also noted in the saline control group (Table 3.3), this could be due to other factors such as the stress of receiving the injection, or it could also be due to stress from caging. It was noted that the nesting was sparse, which could have contributed to the piloerection. This was rectified and all nesting was increased in the cages until the end of the study. this appeared to reduce piloerection, as it was no longer noted later on in the study. The mild piloerection could also be due to the acidity of the MLA. On formulation, the MLA was pH tested and found to be around pH 3.1-3.6. The acidity of the drug could produce some irritation under the skin causing the piloerection. No other behavioural signs were noted in the saline control group,

which was expected. Some mild head-weaving was observed in some rats during the first 90 minutes of the test. One rat appeared slightly subdued in the first 15 minutes of the test and one displayed mild body twitches at 120 minutes post-injection, which disappeared quite rapidly. Overall, there were no major displays of behavioural abnormalities which could confound future studies.

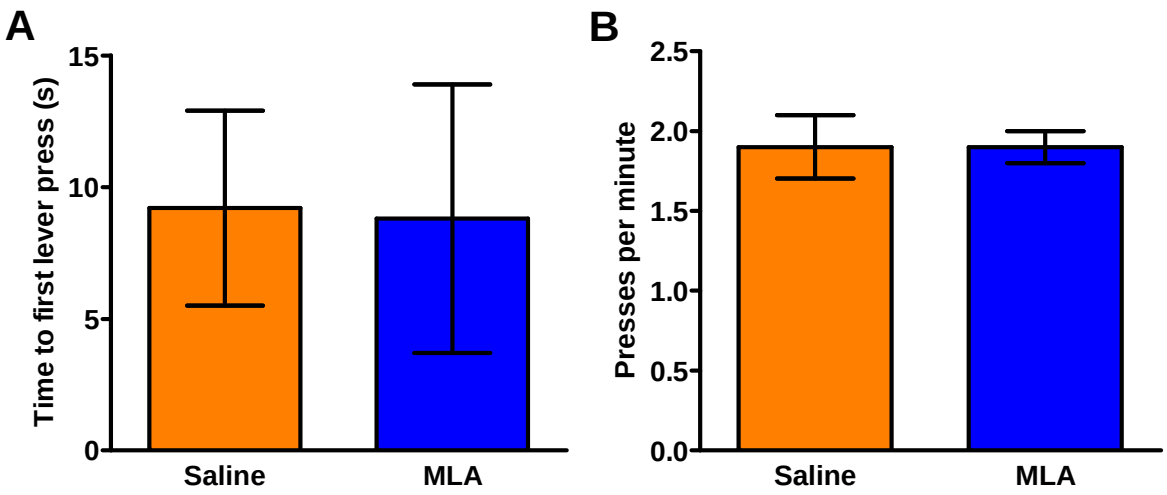


Figure 3.4: **A:** Time to first lever press for food reward in rats treated with either saline (1mL/kg, s.c.) or MLA (4 mg/kg, s.c.) 20 minutes prior to the test session. Unpaired t test, p=0.956 **B:** Rate of lever pressing for food reward in rats treated with either saline (1mL/kg s.c.) or MLA (4 mg/kg s.c.) 20 minutes prior to testing. Unpaired t test, p=0.898. N=6 for both treatment groups.

Lever pressing responses for food reward were recorded during this experiment. There was no difference in the time to first lever press between the MLA and saline-treated animals, showing no effect of MLA on either locomotion or the operant memory (Figure 3.4A). There was also no difference in the rate of lever pressing between treatment groups (Figure 3.4B), demonstrating no effect of MLA on lever pressing behaviour. This preliminary experiment showed there were no

major effects of MLA alone on the overall behaviour of the rats and MLA had no effect on the ability to acquire lever pressing for a food reward.

3.4.2. Heroin self-administration and extinction

Once rats had acquired lever pressing for heroin at a higher training dose (0.05 mg/kg/infusion, see methods section 3.3.7.4), they were trained on an FR5 schedule of reinforcement with 0.015 mg/kg/infusion heroin with contingent tone and light cues. When responding had stabilised (mean of infusions of last 3 sessions ≥12), the means of the last 3 training sessions were taken as measures of acquisition of heroin IVSA (**Figure 3.5**). They then underwent saline extinction in the absence of the tone and light cues until responding had reached the acceptance criterion (mean of active lever presses ≤ 30% of active heroin lever presses) and the means of the last 3 sessions were taken as a measure of extinction. **Figure 3.5** below shows the acquisition of heroin self-administration and extinction of lever pressing in two cohorts of rats, group 1 and group 2. After extinction, each group was assigned a treatment group and received either MLA (4 mg/kg, s.c.) or saline (1 mL/kg, s.c.) 20 minutes prior to subsequent experiments

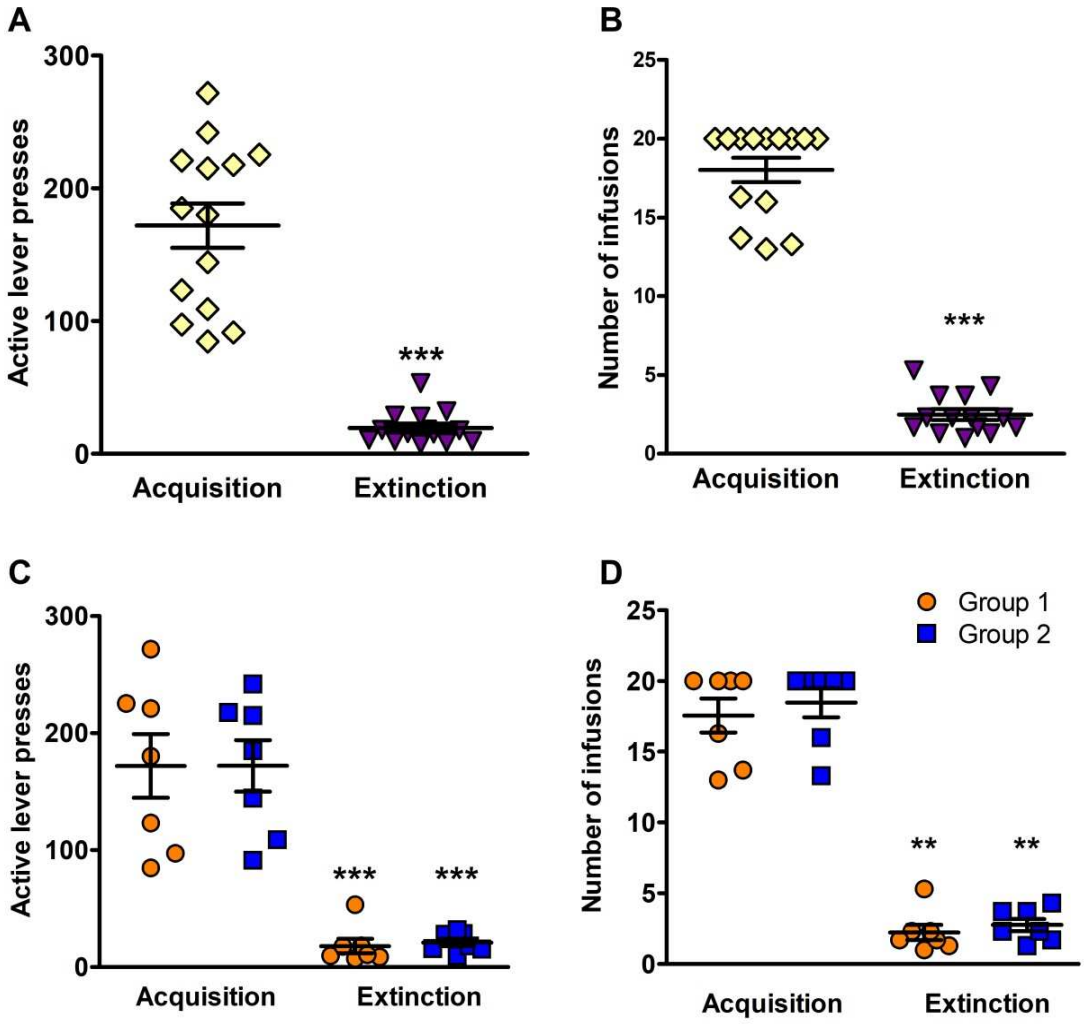


Figure 3.5: Number of active lever presses (**A**) and heroin (0.015mg/kg/infusion) infusions (**B**) during heroin self-administration (acquisition) and extinction in all rats. Rats were then pseudo-randomly assigned to a treatment group (group 1 and 2) by counterbalancing their respective lever pressing and infusion responses (**C** and **D**) for subsequent reinstatement. All data points are the means of the last 3 sessions for individual rats and the group mean±SEM bars overlaid. Kruskal-Wallis test with Dunn's post-hoc analysis, vs acquisition $^{**}p<0.01$, $^{***}p<0.005$. n=7 in both groups. Group 1 was assigned to the saline treatment group and group 2 to the MLA treatment group following extinction for subsequent reinstatement.

After exclusions (see methods Section 3.3.7.4), all rats acquired stable lever pressing for heroin (172±16.8 presses, Figure 3.5A) and the mean number of infusions for all rats was 18±0.8 (Figure 3.5B), which was close to the maximum allowed in a session (20), showing robust acquisition of heroin self-administration.

Following saline extinction, there was a significant decrease in the number of active lever presses and the number of infusions (Figure 3.5A and B), demonstrating successful extinction of lever pressing behaviour. These results showed that rats had stably and robustly acquired heroin intravenous self-administration, and this behaviour was successfully extinguished.

Following extinction, the rats were pseudo-randomly assigned to one of two treatment groups prior to reinstatement, by counterbalancing the lever pressing behaviour and number of infusions. Figure 3.5 C shows there was no significant difference in the mean number of active lever presses between both groups of rats (group 1 mean=171±27 vs group 2 mean=172±22 lever presses). There was also no significant difference in the mean number of infusions between groups (17.6±1.2 vs 18.5±1.0, respectively, Figure 3.5D). During the extinction phase, there was no significant difference in the number of active lever presses or infusions between groups (Kruskal Wallis test group1 vs group 2; active lever presses acquisition: p=1, extinction p=0.28; infusions acquisition: p=0.66, extinction: p=0.33). Figure 3.5C and D showed that both groups were balanced in their responses during acquisition of heroin self-administration and during extinction, allowing an equal comparison of responses during subsequent experiments.

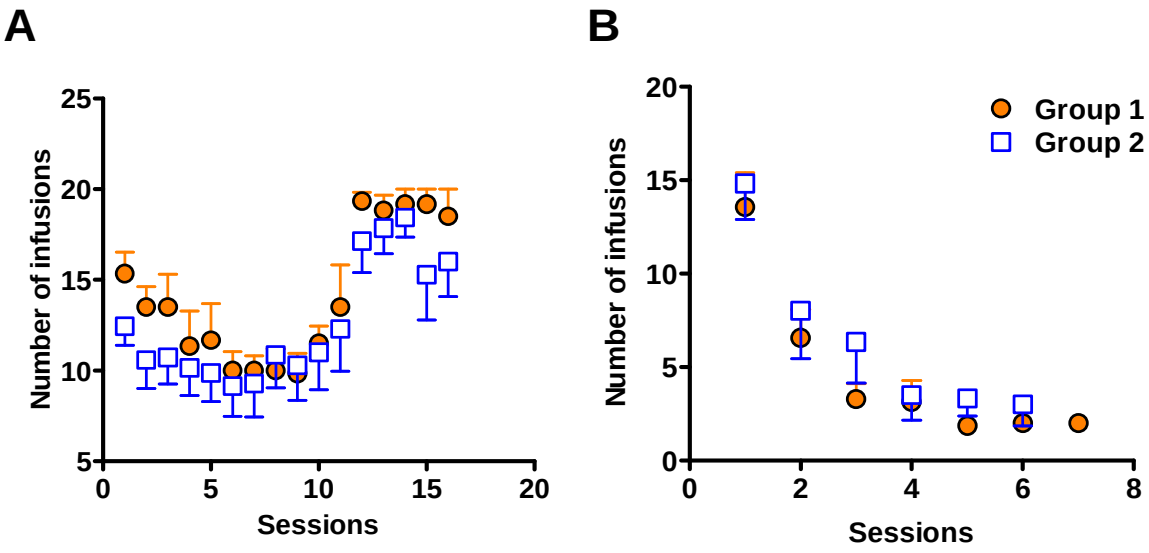


Figure 3.6: Time for both randomly assigned groups to achieve acquisition criteria (**A**) and extinction criteria (**B**). Data are mean±SEM, n=7 per group. After extinction, group 1 was assigned to the saline control group and group 2 to the MLA treatment group.

The majority of rats underwent heroin training for 16 sessions (2 rats required 2-3 additional sessions, data not shown). When observing the ability of the rats to learn the heroin self-administration (i.e. to achieve the acceptance criteria for acquisition of IVSA), both groups had reached the acquisition criteria by session 11, and maintained a high level of responding until the final 16th session (Figure 3.6A). Extinction of lever responding was much quicker and within 4 sessions, the majority of the rats had reached the acceptance criteria for extinction, in both groups (Figure 3.6B). This low response was stable during the subsequent 3 sessions, the means of which were taken as their extinction response.

Session	Treatment	n	Mean (s)	SEM	p vs group 1	p vs extinction
Heroin acquisition	Group 1	7	320.7	56.6		<0.001***
	Group 2	8	255.7	53.4	0.634	<0.001***
Saline extinction	Group 1	7	1524.8	209.8		
	Group 2	8	1210.3	260.1	0.291	

Table 3.5: Inter-injection interval during the acquisition of heroin self-administration and saline extinction. Means were back-transformed from a square root transformation and SEMs were calculated from the residuals of the statistical model. Multiple t test comparing group 1 and group 2 and acquisition to extinction ***p<0.001.

When comparing the mean times between infusions (Table 3.5) during the acquisition and extinction of heroin IVSA between randomly assigned groups, there was no significant difference between groups. There was however a significant (~5-fold) increase in the inter-injection interval in both groups during extinction, demonstrating longer periods of time in between infusions, due to the lack of reward from saline.

These data show that all rats acquired heroin self-administration, and this behaviour was extinguished. The pseudo-random assignment of treatment groups showed both groups behaved equally in both the learning of the lever pressing behaviour and in their responses once the behaviour was acquired. They were also equal in their extinction responses, demonstrating a balanced cohort of rats.

3.4.3. Effect of MLA on the heroin- and cue-primed reinstatement of heroin seeking

Following extinction, group 1 from the pseudo-random division of the cohort was assigned to the saline treatment group and group 2 was assigned to the MLA treatment group. Rats received either saline (1 mL/kg, s.c.) or MLA (4 mg/kg, s.c.) and were placed in their home cages for 20 minutes. Immediately before the beginning of the reinstatement session, all rats received a heroin priming dose (0.015 mg/kg) intravenously. Rats were then immediately placed in the operant chamber and the session was initiated by a non-contingent display of the light and tone cues on an FR5 schedule of reinforcement with saline infusions delivered on the correct number of lever presses (see Table 3.1 for details). The reinstatement sessions were performed over four consecutive days, where the rats were treated with saline or MLA 20 minutes before each heroin and cue-primed reinstatement session.

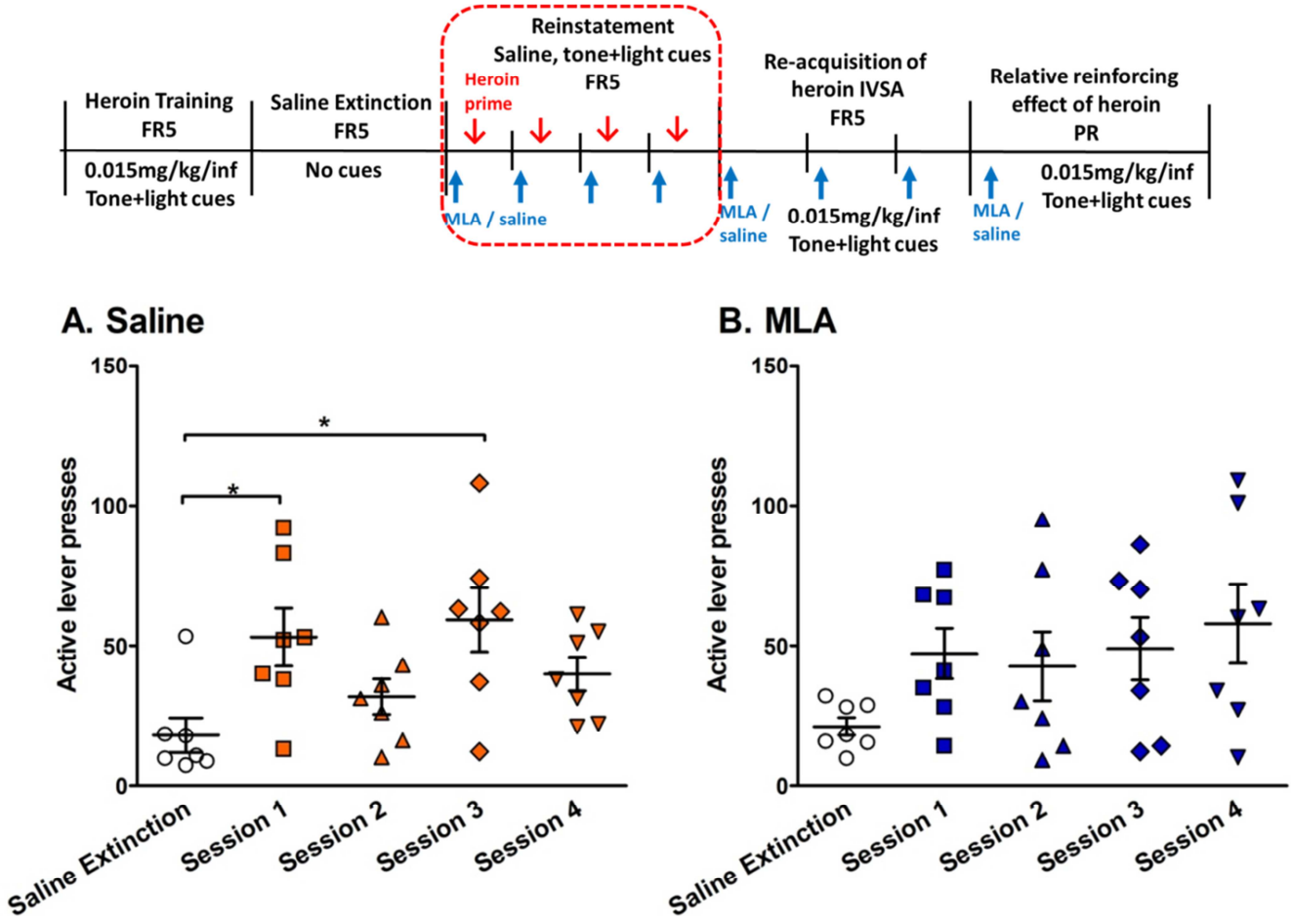


Figure 3.7: Active lever presses of rats pre-treated with **A:** saline (1mL/kg s.c.) or **B**: MLA (4mg/kg, s.c.) 20 minutes prior to heroin and cue-primed reinstatement over 4 sessions (one per day). Data presented as individual rat responses with mean±SEM overlaid. One-way ANOVA with Dunnet's multiple comparison post-hoc analysis vs extinction. *p<0.05, **p<0.005. n=7 for each group.

In the saline control group (Figure 3.7A), there was a significant ~3-fold increase in mean lever responses during the first reinstatement session compared to extinction, demonstrating a significant reinstatement of drug seeking behaviour. During session 2, lever pressing declined to approximately double extinction values and there was no significant difference. During the third reinstatement session, there was again a significant increase in lever responses (~3.5 times extinction presses). In the fourth session, active lever presses declined again to approximately double extinction values.

In the MLA group (Figure 3.7B), during the three first reinstatement sessions, active lever responses doubled compared to extinction, but there was no significant difference. In session 4, there was a ~3-fold increase in lever responses, but due to the variability of the data, this was not significant ($p=0.1$). This suggested there could be a potential effect of MLA on the reinstatement of drug seeking.

During reinstatement, in both treatment groups, it appeared that the variability in responses increased compared to extinction, as some rats showed high responses and others much lower responses in the same session. Interestingly, in the MLA group, there appeared to possibly be two subpopulations of responders: a group of 3 high responders, which showed a ≥3-fold increase in lever responses compared to extinction and a group of 4 low responders, which displayed lever ≤double extinction responses. This could suggest a group of MLA-sensitive rats, which showed a lack of reinstatement, and a group of MLA-insensitive rats, which reinstated to drug seeking.

A direct comparison of individual rats' responses during the acquisition of heroin self-administration and their reinstatement responses was made. Observing the data in this way would allow us to identify whether the highest responders during acquisition would be predictive of the highest responses during reinstatement; and to investigate the possibility of there being subpopulations of responders in reinstatement. Extinction values were omitted for improved clarity.

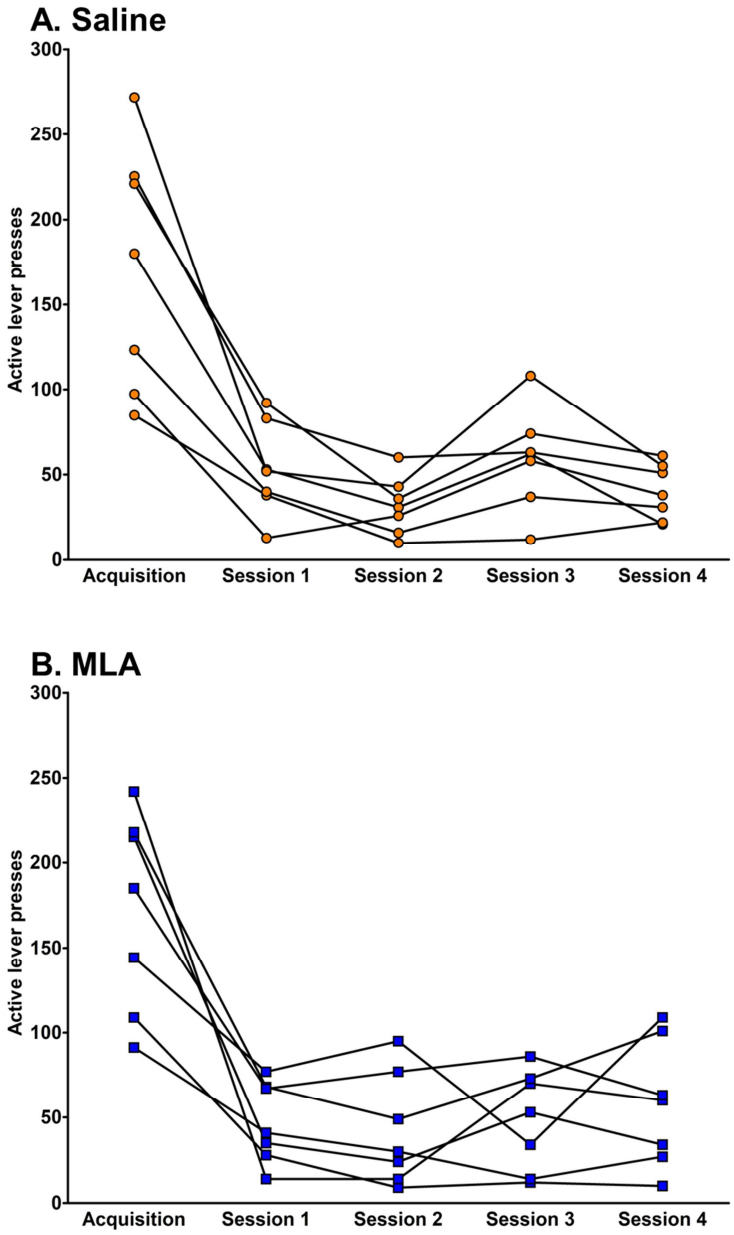


Figure 3.8: Number of active lever presses of individual rats during heroin acquisition and the four drug- and cue-primed reinstatement sessions in the saline (A) and MLA treatment group (B). N=7 per group.

What was made apparent in Figure 3.8 was that during reinstatement, active lever responses were much lower than during acquisition, which was the opposite effect compared to heroin CPP (See Chapter 2, Figure 2.6), where rats spent more time in the drug-paired side during reinstatement than the post-conditioning test. In the saline group (Figure 3.8A), the reinstatement of active lever presses in session 1 was relatively uniform, as shown by the general lack of crossover of the lines; in that the highest and lowest responding rats during acquisition remained the highest and lowest responders during reinstatement. This effect was generally constant during the subsequent reinstatement sessions, as the highest responders during session 1 remained the highest responders during sessions 2, 3 and 4.

In the MLA group however (Figure 3.8B), there was more crossover seen from acquisition to the first reinstatement session, suggesting more variability in the responses. In fact, the two highest responding rats during acquisition in the MLA group showed the lowest lever responses in reinstatement. This increase in crossover from highest to lowest responder suggested that MLA could be having an effect on the reinstatement to lever responding, but that the effect is not consistent across all animals. This needs further investigation as the study is underpowered. Interestingly, in the subsequent reinstatement sessions, the hypothesised high responders (≥3x extinction responses) and low responders (≤2x extinction responses) remained generally constant from the first reinstatement session, with fewer crossovers than the initial session (except session 3).

Due to the variability of the responding observed over the four reinstatement sessions in the saline control group, it was deemed appropriate to focus on the results obtained in the first session, as there was a significant reinstatement of

drug seeking behaviour in the saline group. As the rats received saline infusions during reinstatement, they also acted as extinction sessions, therefore affecting the subsequent reinstatement sessions. In order to determine if there was a relationship between reinstatement behaviour and initial acquisition of heroin IVSA, and to identify possibly subpopulations of responders in the MLA group during reinstatement, correlation analysis was undertaken.

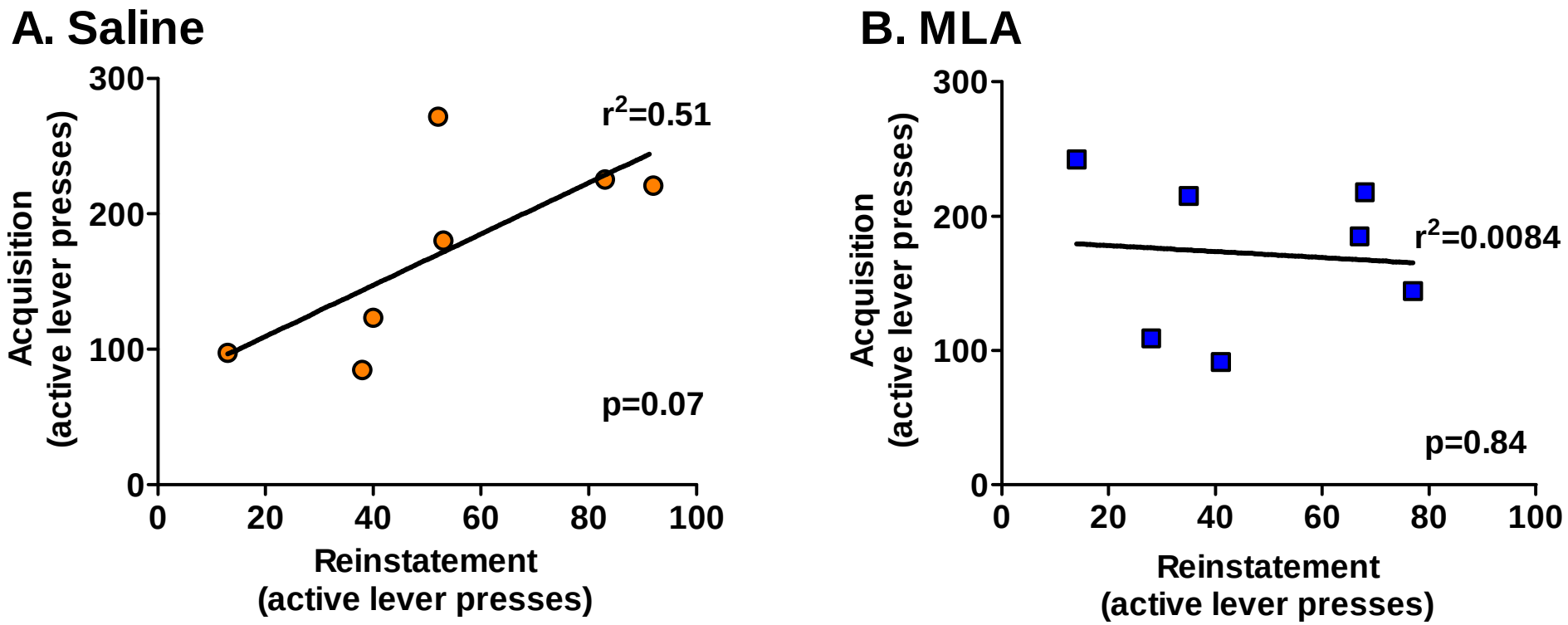


Figure 3.9: Correlation and linear regression of active lever presses during acquisition and the first reinstatement session in the saline group (**A**, n=7) and the MLA group (**B**, n=7). R^2 values of linear regression and p values of correlation are indicated on the graphs.

Correlation analysis of the lever pressing responses during the acquisition of heroin IVSA and the first reinstatement session (Figure 3.9) showed that in the saline control group there could possibly be a correlation between the number of active lever presses during acquisition and those during reinstatement, as the correlation was close to significance (p=0.07). It is therefore possible that the high responders during acquisition of heroin self-administration could be predictive of being high responders during reinstatement, however the study is underpowered and needs additional subjects to confirm this hypothesis.

Although there were no statistically significant differences between the saline and MLA-treated cohorts, this analysis does suggest 2 interesting aspects. First, whereas there is clearly no correlation between number of responses during acquisition and reinstatement in the MLA group (r^2 = 0.0084) there was a trend in the saline-treated group (r^2=0.51). Further, in the MLA group, there appeared to be a group of 4 rats which showed low reinstatement responses, and 3 rats that showed higher reinstatement responses, supporting initial observations. Potentially, this could mean that there is a subpopulation of rats for which MLA had an effect, and another subpopulation where MLA had no effect. Further studies would be required to investigate this possible effect further, as this current study is underpowered.

The responses during the first reinstatement session were compared between treatment groups to determine a potential effect of MLA compared to saline.

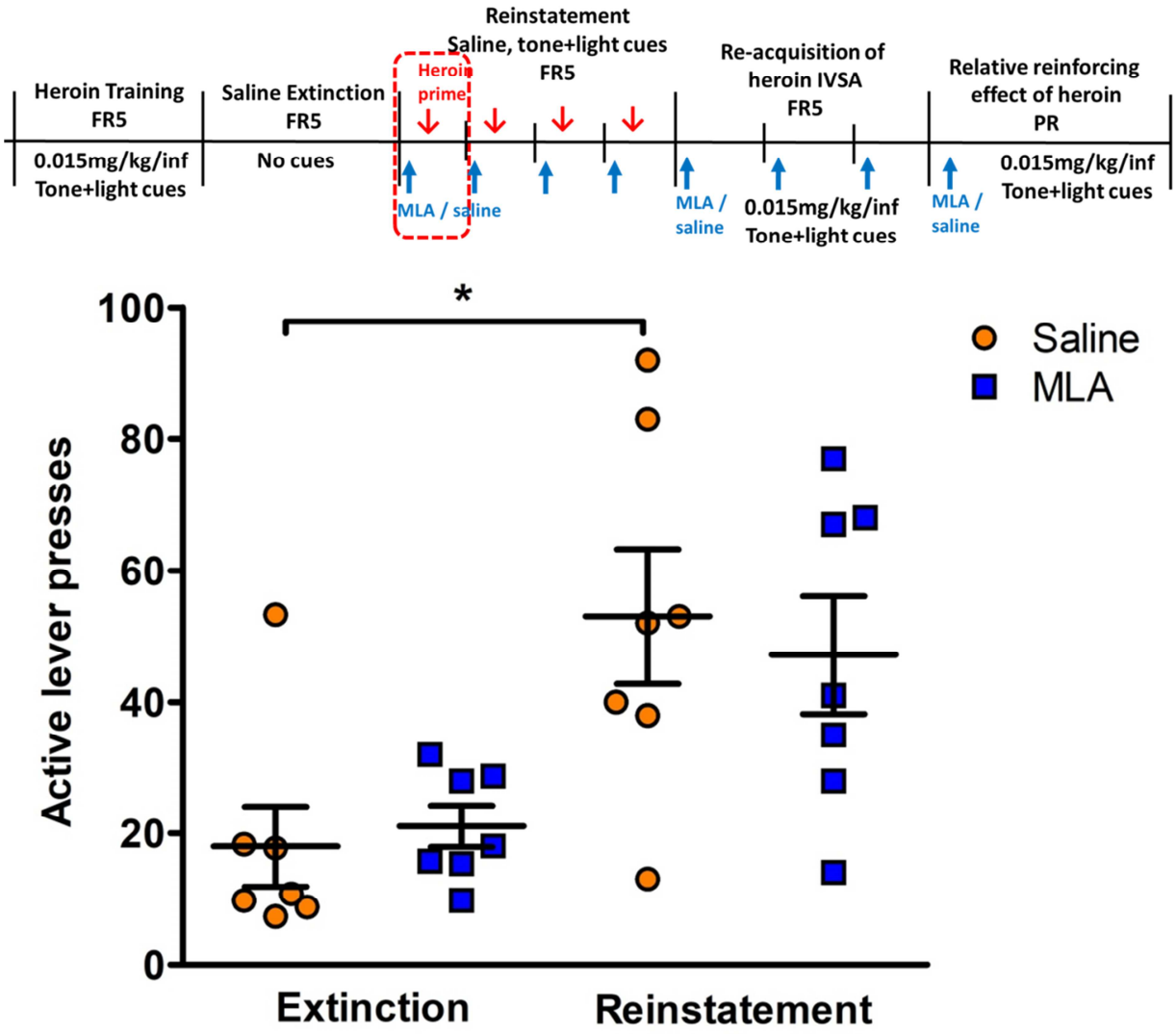


Figure 3.10: Active lever presses for saline reward in rats pre-treated with either saline (orange circles, 1mL/kg s.c.) or MLA (blue squares, 4 mg/kg s.c.) 20 minutes prior to the first reinstatement session. Data points are individual rat responses with mean±SEM overlaid. Two-way ANOVA with Bonferroni post-hoc analysis. *p<0.05, n=7 per group.

Figure 3.10 above shows the comparison of reinstatement responses in the control and MLA treatment groups in the first reinstatement session. Heroin and cue priming significantly increased active lever pressing in the saline control group compared to their saline extinction values, suggesting significant reinstatement of drug seeking. The MLA treatment group showed no significant difference in the number of active lever presses compared to extinction, suggesting a lack of

reinstatement of drug seeking behaviour. There was however no difference in lever pressing between treatment groups during reinstatement. We can therefore neither confirm nor reject the null hypothesis that there is no effect of MLA on reinstatement of drug seeking behaviour.

The time-dependent effect of MLA on the drug- and cue-primed reinstatement of drug seeking was of interest; therefore the data was divided into 5 minute time bins. Keogh et al. (2017) analysed the cue and drug-primed reinstatement of cocaine IVSA in 5 minute time bins and found that there was a significant increase in lever presses within the first hour of the reinstatement session. The results from the present study were therefore divided into 5 minute time bins to observe the time-dependent changes in lever pressing behaviour in saline and MLA-treated rats during reinstatement.

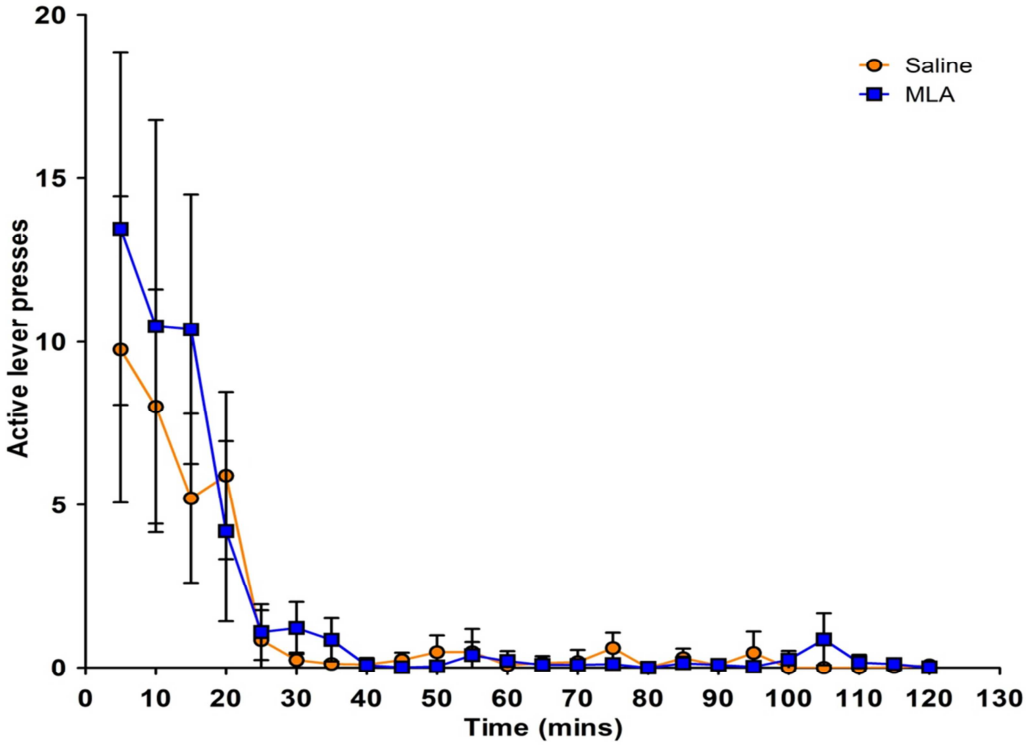


Figure 3.11: Effect of MLA on active lever presses in 5 minute time bins during heroin- and cue-primed reinstatement of IVSA. Data were back-transformed from square root transformation and adjusted for differences between animals. SEMs were calculated from the residuals of the statistical model. Data are mean±SEM, saline: n=7; MLA: n=8. Multiple t test saline vs MLA, not significant.

The analysis of the number of active lever presses in 5 minute increments (Figure 3.11) revealed that only at 20 minutes was there a significant reinstatement of lever pressing compared to extinction in the control group. There was no significant reinstatement in the MLA group in any of the time bins. There was also no difference in lever pressing between treatment groups. This was mostly due to the large variability observed in both data sets. The time bin analysis did however reveal that the majority of the activity during the reinstatement session occurred during the first 30 minutes of the testing session, and that after the first hour of the reinstatement session, lever pressing was close to zero for the remainder of the session. This result was kept in mind for methodological consideration.

Overall, these combined results showed that heroin and cue priming significantly reinstated drug seeking behaviour in the control group, but there was no significant reinstatement in the MLA group. There was however also no difference in the active lever responses between treatment groups, so the null hypothesis that MLA had no effect on the reinstatement of drug seeking could not be rejected. Due to the large variability seen during reinstatement, the study was underpowered therefore additional subjects are needed to complete this study.

Although there were no time-dependent effects of MLA on reinstatement, it was hypothesised that it could affect the initial motivation to seek the reward. The latency to first press was therefore compared during acquisition, extinction and reinstatement, and a potential effect of MLA on these behaviours was investigated.

Session	Treatment	n	Mean (s)	SEM	p vs Saline	p vs Extinction	p vs Acquisition
Acquisition	Saline	7	107.7	37.5		0.984	
	MLA	8	74.9	17.7	0.575	0.903	
Extinction	Saline	7	106.3	28.5			
	MLA	8	68.8	25.9	0.511		
Heroin- and cue-primed reinstatement	Saline	7	33.7	21.4		0.157	0.151
	MLA	8	57.2	18.9	0.567	0.805	0.713

Table 3.6: Time to first lever press (s) during the acquisition, extinction and heroin- and cue-primed reinstatement of IVSA. Means were back-transformed from a square root transformation and SEMs were calculated from the residuals of the statistical model. Multiple t test comparing MLA vs saline in all sessions, reinstatement vs acquisition and reinstatement vs extinction showed no significant effect.

The latency to first press was 107.7±37.5 seconds in the control group during acquisition (Table 3.6). There was no change in the latency to first press during extinction (106.3±28.5 seconds). The latency to first press during reinstatement was lower than during acquisition (33.7±21.4 seconds), though due to variability, this was not significant (p=0.151). In the MLA group, the latency to first press during acquisition was 74.9±17.7 seconds. The latency to first press remained constant throughout saline extinction and reinstatement (68.8±25.9 and 57.2±18.9), showing no change in motivation to seek the lever. Despite a slight decrease in latency to first press in the saline group during reinstatement, there was no difference compared to the MLA group, suggesting MLA had no effect on the initial motivation to seek the lever.

It was also hypothesised that MLA could affect lever pressing behaviour and the time to seek the next reward after infusions. The inter-injection interval was therefore recorded to probe for any potential changes in lever pressing behaviour.

Session	Treatment	n	Mean (s)	SEM	p vs Saline	p vs Extinction	p vs Acquisition
Acquisition	Saline	7	320.7	56.6		<0.001***	
	MLA	8	255.7	53.4	0.634	<0.001***	
Extinction	Saline	7	1524.8	209.8			
	MLA	8	1210.3	260.1	0.291		
Heroin- and cue-primed reinstatement	Saline	7	714.4	219.8		0.004**	0.036*
	MLA	8	412.5	59.0	0.113	<0.001***	0.268

Table 3.7: Inter-injection interval during acquisition, extinction and heroin- and cue-primed reinstatement of IVSA. Means were back-transformed from a square root transformation and SEMs were calculated from the residuals of the statistical model. Multiple t test comparing MLA vs saline (not significant) and reinstatement vs acquisition: *p<0.05, and reinstatement vs extinction: **p<0.005, ***p<0.001.

During extinction, the inter-injection interval was far greater than during acquisition in both treatment groups, signifying a greater amount of time to seek the next infusions (Table 3.7). Heroin- and cue-primed reinstatement significantly decreased the inter-injection interval compared to extinction in both groups, demonstrating an increase in motivation to obtain the reward. Though there was a significant decrease in the inter-injection interval during reinstatement (714.4±219.8 s) in the saline control group compared to extinction (1524.8±209.8), this interval was still significantly higher than during acquisition (320.7±56.6 s). This potentially suggests although the motivation to seek the reward is increased compared to extinction, it is still lower than during acquisition; and as discussed above, the number of active lever presses during reinstatement was also lower than during acquisition (Figure 3.6). This could hint at a methodological flaw which will be discussed further in section 3.5. There was no significant difference in the inter-injection intervals during reinstatement between the saline group and the MLA group (though the time in the MLA group was lower, p=0.113) due to the variability in the control group. The lack of difference in the inter-injection intervals between treatment groups suggests that MLA had no effect on the motivation to obtain infusions during reinstatement.

3.4.4. Effect of MLA on the re-acquisition of heroin self-administration

The next experiment studied the effect of antagonising α7nAChRs on the re-acquisition of drug-seeking behaviour. Following the heroin- and cue-primed reinstatement for saline reward, rats underwent saline extinction sessions to reduce lever pressing responses to again meet the extinction acceptance criteria (section 3.3.7.5). They then remained in their treatment groups and received either

saline (1 mL/kg s.c.) or MLA (4 mg/kg s.c.) 20 minutes prior to being placed in the operant chamber. The 2 hour test session was initiated by a non-contingent infusion of heroin (0.015 mg/kg/infusion i.v.) and non-contingent presentation of the light and tone cues. Rats were on a FR5 schedule of reinforcement with heroin reward (0.015 mg/kg/inf).

As this was an analysis of the re-acquisition of IVSA, the number of infusions was analysed rather than the number of active lever presses, as this signified the number of times the rat successfully pressed for a drug reward. Each rat underwent between 4 and 6 sessions, to stabilise responding and the last 3 re-acquisition sessions were analysed.

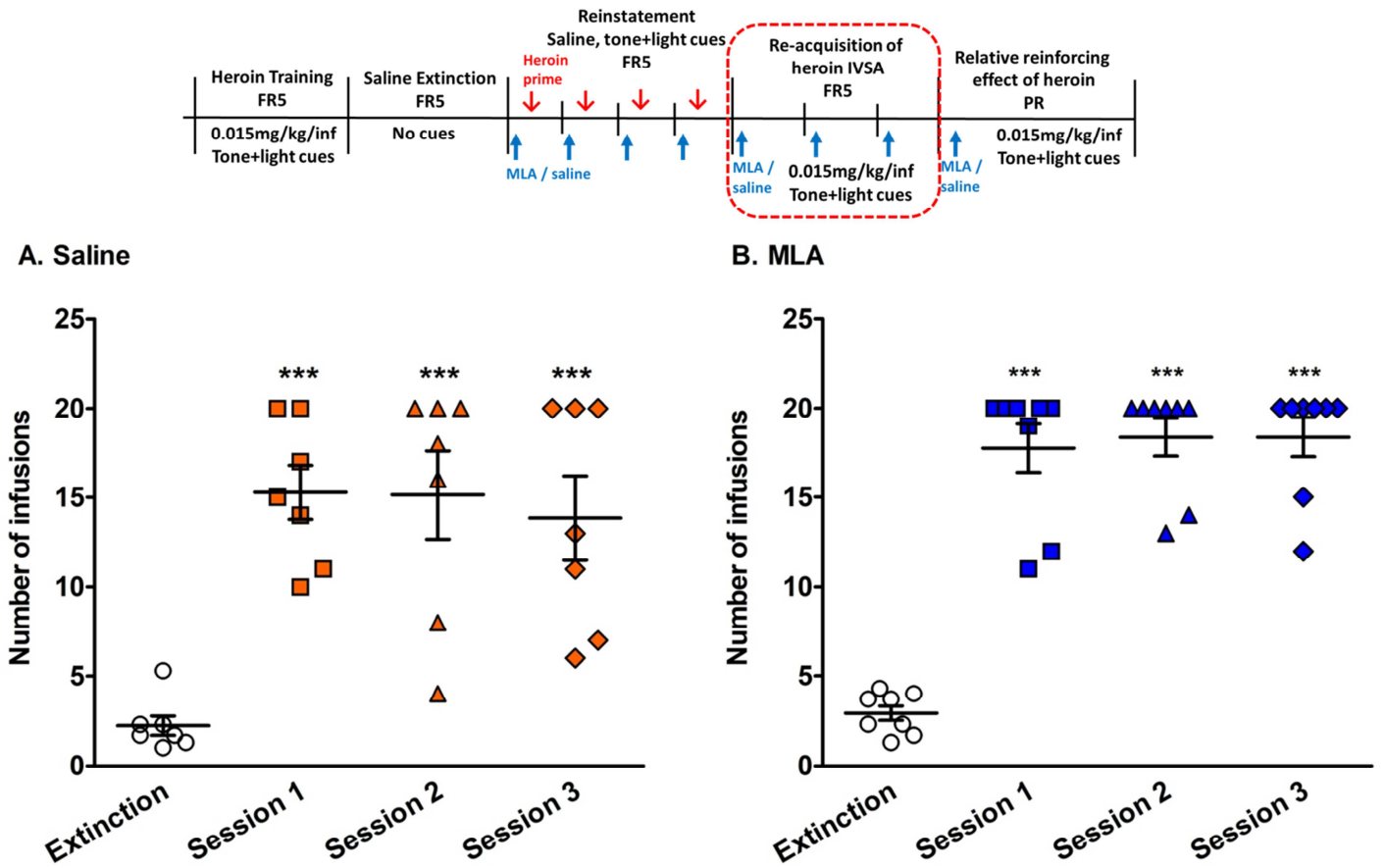


Figure 3.12: Number of heroin infusions in rats pre-treated with **A:** saline (1mL/kg s.c.) or **B**: MLA (4mg/kg, s.c.) 20 minutes prior to the re-acquisition of heroin self-administration over 3 sessions (one per day). Data points are individual rat responses with mean±SEM overlaid. One-way ANOVA with Dunnet's multiple comparison post-hoc analysis vs extinction: ***p<0.001, n=7 per group.

In the saline group (Figure 3.12A), there was a ~5-fold significant increase in the number of infusions taken during all three sessions, compared to extinction. Similarly, in the MLA group (Figure 3.12B), there was a significant, ~6-fold increase in heroin infusions, compared to extinction. This robust increase in heroin infusions suggested that both groups had significantly re-acquired heroin self-administration. Responding was stable across all three sessions, as lower responders (2 in each treatment group) in session 1 remained the lowest responders in sessions 2 and 3. As the responding was stabilised during all three sessions, results from the first reinstatement session were used for comparisons between treatment groups.

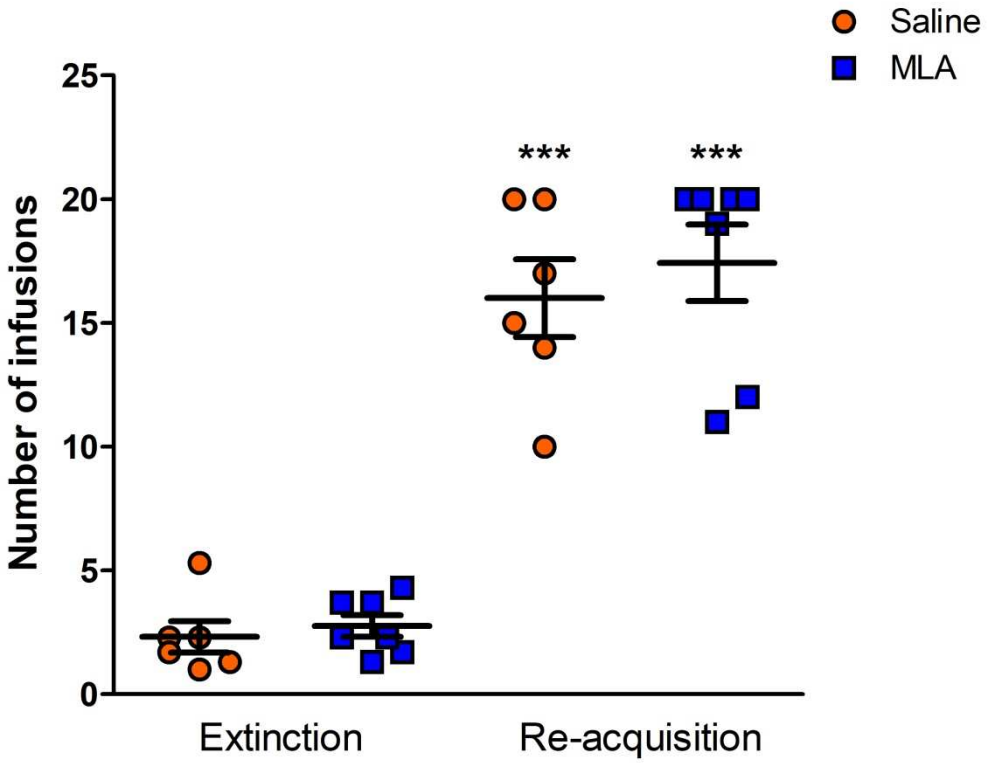


Figure 3.13: Effect of MLA on the first re-acquisition session. Data are individual rat responses with means±SEM overlaid. 2-way ANOVA with Bonferroni post-hoc analysis vs extinction: ***p<0.001, n=7 per group.

As mentioned before, both treatment groups showed a significant increase in the number of infusions taken compared to extinction. Further analysis (Figure 3.13) showed that there was no significant difference in the number of infusions received during re-acquisition between treatment groups, demonstrating that both groups has robustly re-acquired heroin self-administration, and that MLA treatment had no effect on the re-acquisition of this behaviour.

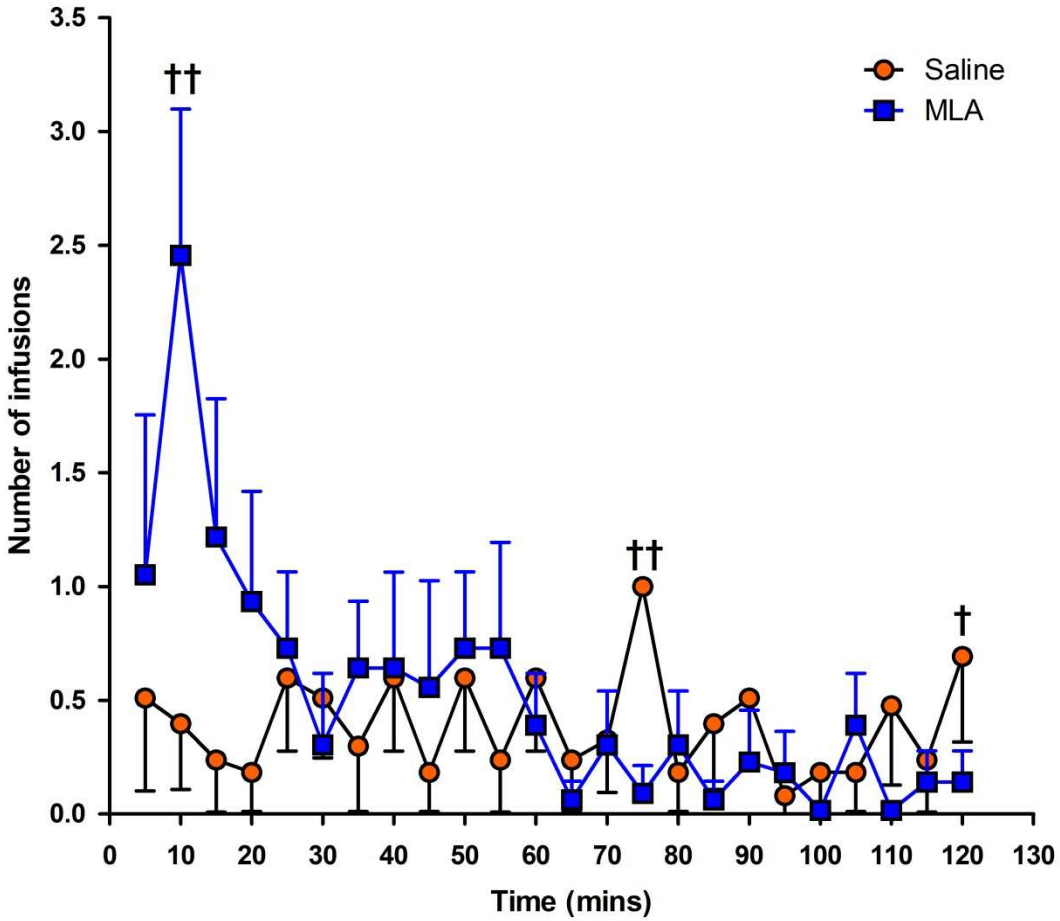


Figure 3.14: Effect of MLA on the number of heroin infusions (0.05 mg/kg/infusion) in 5 minute time bins during the re-acquisition of heroin self-administration. Data were square root transformed and adjusted for differences between animals. Data were analysed by multiple t test, comparing saline vs MLA treatment (black daggers: $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.005$). Data are mean±SEM, saline: n=7; MLA: n=8.

The time-dependent effects of MLA during the re-acquisition of self-administration were also investigated (Figure 3.14). Analysis of the number of heroin infusions in 5 minute time bins showed that the self-administration of heroin was sustained over the 2 hour period, compared to the reinstatement session, where the majority of activity was during the first 30 minutes of the session (Figure 3.11). The MLA group showed a significant increase in infusions at 10 minutes, compared to saline extinction and compared to saline control (10 minute time point, Figure 3.14). From 15 to 70 minutes, there was no difference in responses between the saline- and MLA-treated groups, however, the responses crossed over so that at 75 minutes the responses in the saline-treated group were significantly higher than both the MLA-treated group and to extinction. Responses in the saline-treated group remained higher than those in the MLA-treated group until the end of the 2 hour session. Over the 2 hour session there was a sustained significant increase in heroin responses during re-acquisition in both groups compared to extinction, showing that both groups had re-acquired self-administration behaviour. Overall, there was no significant difference in responding between treatment groups (apart from 3 individual cases at 10, 75 and 120 minutes); therefore it appeared that there was no effect of MLA on the re-acquisition of heroin self-administration.

Session	Treatment	n	Mean	SEM	p vs Saline	p vs Extinction	p vs Acquisition
Acquisition	Saline	7	107.7	37.5		0.984	
	MLA	8	74.9	17.7	0.575	0.903	
Extinction	Saline	7	106.3	28.5			
	MLA	8	68.8	25.9	0.511		
Re-acquisition	Saline	7	274.0	101.7		0.051	0.053
	MLA	8	58.7	13.6	0.004**	0.831	0.737

Table 3.8: Effect of MLA on the time to first lever press during the re-acquisition of drug taking, vs heroin acquisition and vs saline extinction. Means were back-transformed from square root transformation and SEMs were calculated from the residuals of the statistical model. Multiple t test re-acquisition vs acquisition (not significant), re-acquisition vs extinction (not significant), and saline vs MLA: **p<0.01.

In the saline group, there was a large ~2.6-fold increase in the time to first lever press during re-acquisition compared to acquisition (p=0.053) and extinction (p=0.051), suggesting a decrease in the motivation to seek the lever initially (Table 3.8). However, due to the large variability of the latency to first press in this group, this effect was not significant. In the MLA group, the time to first lever press was lower than in the saline group and more consistent across different stages of self-administration (no significant change). During the re-acquisition of heroin IVSA, the time to first lever press in the MLA group was significantly lower than in the saline group (p=0.004, Table 3.8), suggesting the MLA group were faster in retrieving the lever pressing behaviour during re-acquisition compared to the control group. The saline group took approximately 4 times longer than the MLA group to press the lever for the first time; however, the variability was very large in this group. This will be discussed in section 3.5.3.

To determine any further differences in lever pressing behaviour between treatment groups, the inter-injection interval was also studied.

Session	Treatment	n	Mean (s)	SEM	p vs Saline	p vs Extinction	p vs Acquisition
Acquisition	Saline	7	320.7	56.6		<0.001***	
	MLA	8	255.7	53.4	0.634	<0.001***	
Extinction	Saline	7	1524.8	209.8			
	MLA	8	1210.3	260.1	0.291		
Re-acquisition	Saline	7	446.3	66.8		<0.001***	0.439
	MLA	8	283.3	68.6	0.287	<0.001***	0.829

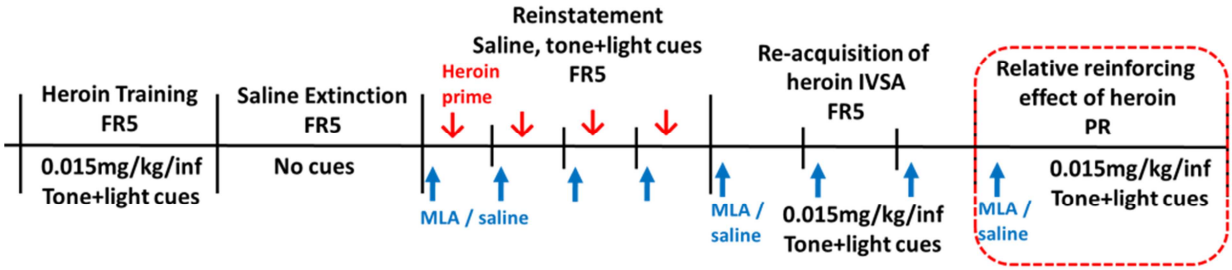
Table 3.9: Inter-injection interval comparing re-acquisition of heroin IVSA to acquisition and saline extinction and between treatment groups. Means were back-transformed from a square root transformation and SEMs were calculated from the residuals of the statistical model. Multiple t test comparing reacquisition vs extinction: ***p<0.001, reacquisition vs acquisition (not significant) and saline vs MLA (not significant).

The inter-injection interval recorded during the re-acquisition of heroin self-administration showed that in both treatment groups, the times between infusions were similar to the times during the acquisition of heroin IVSA (Table 3.9). This result showed that in both groups, the lever pressing behaviour was restored and the motivation to obtain the heroin reward was the same as during the initial acquisition of the behaviour. There was also no difference during re-acquisition between treatment groups, supporting the finding that MLA had no effect on the re-acquisition of heroin intravenous self-administration (Figure 3.13).

The combined results show that overall; MLA had no effect on the re-acquisition of heroin IVSA, as both saline and MLA-treated rats robustly re-acquired heroin

taking behaviour. In order to probe this finding further, the effect of MLA was tested on the relative reinforcement of heroin in a progressive ratio session.

3.4.5. Effect of MLA on the relative reinforcement of heroin



In order to observe the effects of MLA on individual rats' motivation to receive a heroin infusion, they underwent a single progressive ratio session after the final re-acquisition session. Rats remained in their treatment groups and received either saline (1 mL/kg s.c.) or MLA (4 mg/kg s.c.) 20 minutes prior to being placed in the operant chamber. The testing session was initiated by a non-contingent infusion of heroin (0.015 mg/kg/infusion i.v.) with a simultaneous presentation of the tone and light cues. The test session was a minimum of 2 hours and the testing session was terminated either if rats had not pressed the active lever for 30 minutes, or if 4 hours had elapsed. The schedule was progressively increased with each infusion (see section 3.3.7.8 for progressive ratio lever pressing schedule), so as to determine the motivation to receive a single infusion for each individual rat. This was determined by comparing the breakpoints of each treatment groups.

Treatment	n	Mean	SEM	p vs saline s.c.
Saline s.c.	7	48.4	15.2	
MLA s.c.	8	94.7	32.4	0.201

Table 3.10: Effect of MLA on lever pressing breakpoint. Means were back-transformed from square root transformation and SEMs were calculated from the residuals of the statistical model, unpaired t test.

The breakpoint responses for the MLA group were approximately double that of the control group, but with a higher SEM (

Table **3.10**). For this reason, there was no difference in breakpoints between the treatment groups (p=0.201), suggesting MLA had no effect on the reinforcing properties of heroin. The latency to first lever press was then investigated to probe for any effects on the motivation to seek the reward.

Session	Treatment	n	Mean	SEM	p vs Saline	p vs Extinction	p vs Acquisition
Acquisition	Saline	7	107.7	37.5		0.984	
	MLA	8	74.9	17.7	0.575	0.903	
Extinction	Saline	7	106.3	28.5			
	MLA	8	68.8	25.9	0.511		
Relative reinforcement of heroin (PR)	Saline	7	212.9	76.6		0.179	0.185
	MLA.	8	119.5	28.5	0.235	0.375	0.444

Table 3.11: Effect of MLA on the time to first lever press during the relative reinforcement of heroin (progressive ratio, PR session), vs acquisition and vs extinction. Means were back-transformed from square root transformation and SEMs were calculated from the residuals of the statistical model. Multiple t test vs acquisition, vs extinction, and saline vs MLA (not significant).

In the saline group, the time to first lever press doubled compared to acquisition and extinction; however, due to large variability in this group, this effect was not significant ($p=0.235$, Table 3.11). In the MLA group, the time to first lever press was slightly higher during the progressive ratio session compared to acquisition and extinction, though there was no significant difference ($p=0.444$ and 0.375, respectively). There was no effect of treatment on the time to first lever press during the progressive ratio session, therefore both treatment groups showed similar motivation to press the lever at the beginning of the session. These results suggest that MLA had no effect on the relative reinforcing effects of heroin, or on the motivation to seek the reward.

3.5. Discussion

This chapter describes experiments designed to examine the effects of MLA on heroin intravenous self-administration (IVSA). MLA was initially tested on lever pressing for food reward and then tested on the heroin- and cue-primed reinstatement and the re-acquisition of heroin IVSA. MLA had no significant effect on any measures examined. The summary of results, methodology used, underlying mechanisms and implications for α7nAChRs will be discussed here.

3.5.1. MLA had no effect on the rewarding effects of heroin

Primary reinforcement of rewards: Behaviour studied	**Effect of MLA vs saline?**	**Figure number**
Lever pressing for food reward	No effect	**Figure 3.4**
Relative reinforcement of heroin (PR)	No effect	**Table 3.10**
Time to first press (PR)	No effect	**Table 3.11**

There was no effect of MLA on lever pressing behaviour for a food reward (Figure 3.4). Furthermore, MLA also had no effect on the relative reinforcing effects of heroin, shown by the lack of change in breakpoints (Table 3.10) and the time to first lever press (Table 3.11) during the progressive ratio session. These combined data strongly suggest that α7nAChRs do not play a role in modulating the

rewarding effect of food or heroin. The results in the previous chapter of this thesis showed no effect of MLA on the acquisition of heroin CPP in rats, and this was supported by the study conducted by Wright et al. (2018), who showed no effect of MLA on the acquisition of morphine CPP in mice and rats. Together with the data shown in this chapter, this strongly suggests that MLA does not affect reinforcement produced by heroin, nor does it affect the animals' abilities to acquire drug-seeking behaviour.

These data are also in agreement with other studies. Liu (2014) showed α7nAChRs played no role in modulating the primary reinforcing effects of nicotine by a lack of effect of MLA on nicotine IVSA in rats. In addition, antagonising α4β2nAChRs blocked the acquisition but not the reinstatement of nicotine IVSA in rats (Liu et al., 2007, Liu, 2014), further demonstrating a selective role of nAChR subtypes in the different stages of addiction. Furthermore, mouse knockout studies have supported these findings that β2-containing nAChRs modulate the primary reinforcing effects of nicotine and α7nAChRs modulate the reinstatement of nicotine seeking (Walters et al., 2006b, Pons et al., 2008, Cahir et al., 2011, Madsen et al., 2015). Secci et al. (2017) demonstrated that kynurenic acid, acting as a negative allosteric modulator of α7nAChRs, inhibited both drug- and cue-primed reinstatement of both nicotine and cocaine self-administration in squirrel monkeys. These data further demonstrate a differential and selective role of nicotinic receptor subtypes in drug reinforcement and support the findings in both this chapter and in Chapter 2.

In contrast, other studies disagree with this and have shown that α7nAChRs are involved in modulating the rewarding effects of nicotine (Markou and Paterson, 2001, Besson et al., 2012); however some of these effects could be due to

α7nAChRs in the VTA (Harenza et al., 2014), rather than the hippocampus, hypothesised in this thesis to mediate the effects of MLA on reinstatement. Knocking out α7nAChRs in the VTA in mice also reduced nicotine intra-VTA administration, suggesting α7nAChRs in the VTA may mediate nicotine reward (Besson et al., 2012). One study suggested that β2-containing nAChRs may regulate the initial nicotine consumption, but may regulate longer-term consumption of nicotine (Levin et al., 2009). The hypothesis of this thesis is that α7nAChRs in the ventral hippocampus modulate the reinstatement of opioid seeking behaviour, but their role with nicotine may be different.

3.5.2. MLA had no effect on the heroin- and cue-primed reinstatement of drug seeking

Heroin- and cue-primed reinstatement: Behaviour studied	**Saline group vs extinction**	**MLA group vs extinction**	**Effect of MLA vs saline?**	**Figure number**
Number of active lever presses	Increase	No change	No effect	**Figure 3.10**
Correlation of acquisition and reinstatement	Trend (p=0.07)	No correlation	-	**Figure 3.9**
5 minute time bins	No change	No change	No effect	**Figure 3.11**
Time to first lever press	No change	No change	No effect	**Table 3.6**
Inter-injection interval	Decrease	Decrease	No effect	**Table 3.7**

Heroin- and cue-priming significantly increased the number of active lever presses in the saline group, showing the reinstatement of drug seeking behaviour in this treatment group (Figure 3.10). Furthermore, the decrease in the inter-injection interval compared to during extinction phase suggests the re-activation of the rats' motivation to seek the reward. In contrast, in animals pre-treated with MLA, heroin- and cue-induced reinstatement of IVSA was not significantly different to during the extinction phase. Potentially, this could suggest that antagonising α7nAChRs inhibited drug- and cue-induced reinstatement, however, as lever-pressing in the MLA-treated group was not significantly different compared to the saline-treated

group, we could neither accept nor reject the null hypothesis that MLA has no effect on the heroin- and cue-primed reinstatement of IVSA. The results are equivocal, and further work is needed to elucidate the effect of MLA on reinstatement, especially by increasing the number of rats due to large variability. The findings in this section do not corroborate the results of the study performed by (Liu, 2014), who showed that MLA inhibited the cue-primed reinstatement of nicotine IVSA.

As previously discussed (Introduction, sections 1.3.4.1 and 1.3.4.2), studies into the pathways involved in the reinstatement of IVSA have shown the recruitment of different brain regions in response to either drug or cue-primed reinstatement (Bossert et al., 2004, Weiss, 2005, Rogers et al., 2008, Namba et al., 2018). There appears to be a distinct role of the hippocampus in cue-induced reinstatement, which has not been observed in drug-primed reinstatement (Rogers et al., 2008, Namba et al., 2018). Nirogi et al. (2012) showed by mass spectrometry that following i.v. administration of MLA in rats, the majority of MLA was detected in the hypothalamus (whole tissue dissected). The second highest concentrations of MLA were found in the hippocampus and striatum, regions of interest for the present study. Furthermore, Wright et al. (2018) showed that only intracerebral infusions into the ventral hippocampus (though only the PFC and hippocampus were examined) inhibited the reinstatement of morphine CPP in rats, suggesting the primary site of action of MLA in inhibiting the reinstatement of CPP is the ventral hippocampus (Wright, 2016, Wright et al., 2018). Based on this evidence, it could be hypothesised that MLA treatment would have an effect on cue- but not drug-primed reinstatement of IVSA, due to the selective activation of the ventral hippocampus.

Drug priming recruits neurons located in the VTA, NAc, mPFC, VP and PPT (Namba et al. (2018), section 1.3.4.2) whereas cue-priming recruits these regions as well as additional regions including the BLA and hippocampus (Rogers et al. (2008), section 1.3.4.1). The present study used a combination of drug and cue priming as this has been shown to have an additive effect on the reinstatement of lever responding with other drugs of abuse (Shelton and Beardsley, 2008, Fattore et al., 2010, Keogh et al., 2017). This increased reinstatement response could suggest the recruitment of additional circuits involved in mediating drug-primed reinstatement, which do not involve the hippocampus (Stewart, 2008, Namba et al., 2018). It is possible that the slight decrease in active lever presses caused by MLA in the reinstatement of drug seeking is due to the antagonism of α7nAChRs in the hippocampus in response to cue-primed reinstatement, but that the recruitment of further neurons in regions such as the mPFC, NAc, VTA, LDT and VP by drug priming had an overshadowing effect on the effect of MLA on lever responding. The only study observing the effect of MLA on the reinstatement of IVSA was performed by Liu (2014). They showed that MLA significantly inhibited cue-induced reinstatement of nicotine IVSA in rats in a dose-dependent manner (2.5 and 10 mg/kg, i.p.), however the higher dose was 2.5 times that used in this thesis. They did not study drug-primed reinstatement therefore further investigation into any differential effects of MLA on these different types of reinstatement is required.

There are several potential explanations to the wide variability in the lever pressing behaviour in the MLA group during reinstatement. The first explanation could be that MLA has no effect on drug- and cue-primed reinstatement of IVSA and that its effect is specific to CPP. The differences in methodology will be discussed further

below. Another explanation is that there could be subgroups of rats that show either weak or strong reinstatement of drug-seeking behaviour regardless of acquisition behaviour. Brown et al. (2010) found that despite acquiring robust cocaine CPP, a subgroup of mice did not exhibit reinstatement in response to a cocaine prime. There were specific differences in brain region activation in these mice; implicating different patterns of brain activation could be indicators of reinstating and non-reinstating mice. This suggests that individual animals could exhibit different reinstatement propensities which could explain the variability in reinstatement responses; however this has yet to be demonstrated with heroin. The correlation analysis of lever responses during IVSA acquisition and reinstatement in the saline control group suggests that this explanation is however unlikely, as there did not appear to be distinct groups of responders in the saline group. In fact, it showed there could be a relationship between responses shown during acquisition and those during reinstatement and that strong responders during acquisition were predictive of being strong responders during reinstatement.

Another explanation is potentially that there was a group of rats that were sensitive to MLA pre-treatment, which showed weak reinstatement and a group of rats that was insensitive to MLA, which showed stronger reinstatement of drug seeking. This is reinforced by the fact that the correlation analysis of responses during IVSA acquisition and reinstatement showed a complete lack of correlation in this treatment group, compared to a trend in the saline group. Furthermore, there appeared to be a group of high responders and a group of low responders in the MLA group regardless of responses during the acquisition of heroin IVSA; potentially suggesting MLA had an effect on one group and no effect on the other.

This could potentially reflect a difference in α7nAChR expression, receptor function or desensitisation between individual rats, which has not yet been investigated.

3.5.3. MLA had no effect on the re-acquisition of heroin self-administration

Re-acquisition of heroin SA **Behaviour studied**	**Saline vs extinction**	**MLA vs extinction**	**MLA vs saline**	**Figure number**
Number of infusions	Increase	Increase	No effect	**Figure 3.12**
5 minute time bins	Some change	Some change	Some effect	**Figure 3.14**
Time to first lever press	No change	No change	Decrease	**Table 3.8**
Inter-injection interval	Decrease	Decrease	No effect	**Table 3.9**

Results from this study showed a robust re-acquisition of heroin self-administration in both treatment groups, shown by the significant increase in heroin infusions and the significant decrease in the inter-injection interval, compared to extinction. There was no effect of MLA pre-treatment on any of these behaviours. This suggests MLA pre-treatment had no effect on the re-acquisition of heroin IVSA. To my knowledge, there are no reports on the effect of MLA on re-acquisition of self-

administration. In CPP, Wright et al. (2018) showed that MLA had no effect on the expression of morphine CPP in mice, where after the acquisition of morphine CPP, mice were treated with MLA 20 minutes prior to the post-conditioning test, for 4 consecutive days following the post-test, and one week later. In all cases, mice spent significantly more time in the drug-paired compartment and there was no difference in preference between sessions (Wright et al., 2018). This showed that MLA had no effect on the ability to re-express the previously acquired association. This experiment closely resembles the re-acquisition of heroin IVSA in this study; therefore the data in this chapter are in agreement with the morphine and heroin CPP acquisition data, where MLA was without effect. These results further support the notion that α7nAChRs do not modulate the primary reinforcing effects of heroin and there is a potential selective role of α7nAChRs in the reinstatement of drug seeking behaviour over the reinforcing properties of heroin.

The time to first lever press was significantly lower in the MLA group compared to the saline-treated group during the re-acquisition of heroin IVSA. This could be interpreted as the MLA group showing a stronger motivation to seek the heroin at the start of the session than the saline-treated group. There was however no difference in the time to first lever press during acquisition, extinction or heroin- and cue-primed reinstatement in either treatment group, therefore it seems like this difference in the saline group is likely coincidental. Furthermore, both groups showed a decrease in inter-injection intervals during the re-acquisition, compared to extinction, and there was no difference in the intervals between treatment groups, suggesting that rats had similar motivations to obtain the rewards during the session. Overall, it seems like the aberrant responses in the saline group

account for the differences in the time to first lever press compared to the MLA group during the re-acquisition of heroin IVSA.

3.5.4. Methodological Considerations

3.5.4.1. The study was underpowered

A major factor affecting the results in this chapter is that the study was underpowered. Initially, there were 26 rats in the study but a large number of rats were excluded from the study due to complications with catheters post-surgery and due to not meeting the acceptance criterion for the acquisition of heroin IVSA. A power analysis was performed prior to this study based on previous work at Renasci on the combination of drug- and cue priming on the reinstatement of cocaine seeking (Keogh et al., 2017). It was calculated that for 80% power to detect an effect of MLA, n=9 per treatment group was needed. Initially, the study began with 26 rats, or n=13 per group, which would theoretically have been sufficient. After exclusions, 14 rats remained, leaving just n=7 per treatment group. Due to the unexpected loss of ~40% of rats during acquisition and extinction, the study was underpowered.

The drug- and cue-primed reinstatement of drug seeking is still of particular interest as, unlike during re-acquisition, the effect of MLA on reinstatement is still equivocal. In both treatment groups, the variance was greatly increased during reinstatement compared to acquisition and extinction and has been previously reported in IVSA (Stewart, 1983, Shalev et al., 2002) and in CPP experiments (Do Couto et al., 2003, Tzschentke, 2007).

3.5.4.2. Food before training and other parameters

In many self-administration studies, animals are often food trained prior to the transition onto drug training. It has been argued that the introduction of food in the training protocol can add confounds to reinstatement (Shalev et al., 2002). For instance, when drug training succeeds food training in the same experimental setup, it has been argued that the drug training comprises a component of extinction training for food; therefore reinstatement of lever pressing post-extinction could be due to the reinstatement of food seeking rather than drug. This has rarely been controlled for, however a counterargument for this is that the food training was performed in the absence of the tone and light cues, and the drug training was performed in the presence of these cues. It could therefore be disputed that the presentation of the contingent cues with the drug marks this association specifically for the rewarding effects of the drug rather than the food. The presentation of the cues during reinstatement should therefore recall only the drug-associated lever pressing rather than lever pressing for food. In addition, in this study, rats were minimally trained with food to prevent strong associations of the lever with food which could confound the study.

3.5.4.3. Drug withdrawal

A methodological component which appears to have a major impact on reinstatement is the drug withdrawal period prior to reinstatement. Studies with cocaine have shown that cocaine- or cue-priming induced significant reinstatement of drug seeking behaviour, but this reinstatement response was greatly increased with increasing periods of withdrawal from cocaine compared to either one week or one day in rats (Tran-Nguyen et al., 1998, Grimm et al., 2001). A study by

Shalev et al. (2001) found that reinstatement of lever pressing behaviour by foot-shock followed an inverted U relationship with the period of heroin withdrawal in rats, with 6 and 12 days withdrawal showing maximal responding. Similar results were found by Doherty and Frantz (2012), who found that rats showed greater cue-primed reinstatement of drug seeking following 12 days of heroin abstinence compared to one day of abstinence. Interestingly in this study, the reinstatement lever responses were much lower than during the acquisition of heroin IVSA; which is reflected in this thesis. Often, these studies found a lack of reinstatement on the first day of withdrawal. Furthermore, a study found that the type of extinction impacts heroin seeking (Venniro et al., 2017). The study found that only forced abstinence (removing subjects from drug self-administration environment) from heroin resulted in significant reinstatement after 21 days (but not one day) of abstinence in rats, compared to voluntary abstinence (discrete choice between drug or palatable food) (Venniro et al., 2017). These results must be taken with caution as analysis of their individual data revealed that only two of the rats showed high responses which drove the significant difference. In the present study, rats were extinguished for a minimum of 4 days before reinstatement, so perhaps increasing the time of withdrawal in this study could increase reinstatement responses.

3.5.4.4. Combination of drug- and cue-priming

A criticism of the methodology was that heroin- and cue-priming did not appear to produce as marked effects on reinstatement to lever pressing as previously reported with other drugs of abuse (Shelton and Beardsley, 2008, Fattore et al., 2010, Keogh et al., 2017). One of the adjustments that was made during this study

was that the priming dose was lowered from 0.05 mg/kg i.v. to 0.015 mg/kg i.v. With the higher dose of heroin, the rats did not appear as active, were slightly too sedated and not motivated enough to press the lever and responding during reinstatement was low. It was inferred that this dose of heroin was too rewarding therefore the animals would require a longer interval before seeking a further infusion after being placed in the operant chamber. When the dose was lowered to 0.015 mg/kg i.v., the animals were much more active in the chamber and the time to first lever press was reduced compared to that of the rats on the higher dose. Those rats that had received a higher heroin priming dose were then re-trained on the lower dose of heroin and re-extinguished to then receive the lower priming dose of heroin.

3.5.4.5. Route and dose of heroin prime

The route of administration of the heroin priming dose (0.015mg/kg, i.v.) could also be a reason for the lower reinstatement response than expected. The intravenous route is a way to produce an acute, short-lasting euphoric effect, which to a certain extent, triggered active lever pressing during the first 30 minutes of the reinstatement session. However, the reinstatement responses were still lower than during acquisition. Gottås et al. (2013) found that after i.v. infusion of 1.3 mg heroin, blood heroin levels peaked immediately, and were then cleared after approximately 10 minutes. They also measured brain extracellular fluid concentrations of heroin and found heroin brain plasma levels also peaked approximately 2 minutes after injection, then levels cleared after 5 minutes, demonstrating the rapid effects of heroin. Heroin's metabolite, 6-monoacetylmorphine (6-MAM), however is detectable in the brain ECF for up to 40

minutes, therefore intravenous administration could still be an acceptable route for heroin priming.

In order to address the issue of the route of heroin administration, at the end of this IVSA study, a small pilot study was performed to test the lever pressing responses of the rats to a subcutaneous priming dose of heroin (**Figure 3.15** below). Animals were re-extinguished, then given a single heroin priming dose (0.25 mg/kg, s.c.) immediately before being placed in the operant chamber and the presentation of the tone and light cues. Lever pressing was on an FR5 schedule of reinforcement with saline infusions. During this testing period, the rats appeared motivated to press the lever and there was a significant increase in lever presses compared to extinction. The number of active lever presses in this preliminary experiment was much higher than during i.v. heroin- and cue-primed reinstatement of drug seeking (149.2±34.7 s.c. vs 53±27.1 i.v.); suggesting subcutaneous heroin priming could be an improvement to the experimental design. This could be due to the subcutaneous route producing a slower and/or more sustained level of heroin into the brain, which could prolong the motivation to seek the drug.

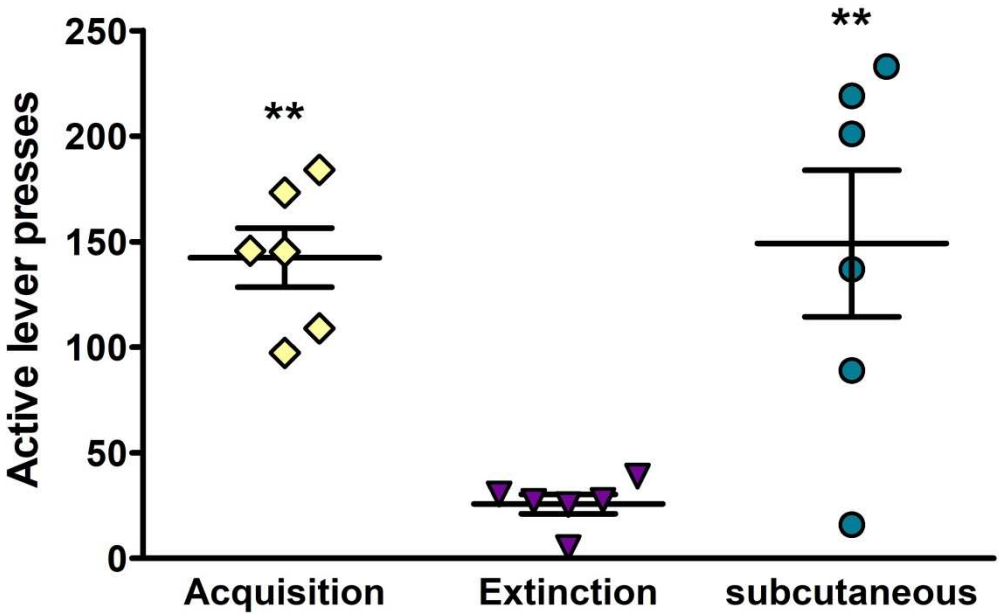


Figure 3.15: Preliminary experiment studying the effect of subcutaneous heroin- (0.25 mg/kg, s.c.) and cue-primed reinstatement of IVSA. Rats from both treatment groups (saline and MLA) were taken at the end of the study for this preliminary experiment. Data points are individual rat responses with mean±SEM overlaid. One-way ANOVA with Dunnet post-hoc analysis vs extinction: **p<0.01, n=6.

3.5.4.6. Dose of MLA

In the previous chapter of this thesis, there was a clear and total inhibition of heroin-primed reinstatement of CPP by MLA, similar to effects also shown by Feng et al. (2011) and Wright et al. (2018), who showed a significant attenuation of morphine-primed reinstatement of CPP in mice and rats (respectively). Intracerebral delivery of MLA into the ventral hippocampus however resulted in a total abolition of the response (Wright et al., 2018), potentially reflecting a larger local dose that fully antagonises α7nAChRs in this region. The systemic dose of MLA could therefore be suboptimal for heroin IVSA, as Liu (2014) found a dose-dependent effect of MLA (2.5 and 10 mg/kg, i.p.) on cue-induced reinstatement of nicotine IVSA. Increasing the dose of MLA does however pose certain risks, as

MLA displays non-specific effects at other nicotinic receptor subtypes (notably α6β2 nAChRs, Mogg et al. (2002)). There are however negligible levels of α6-containing nAChRs in the hippocampus (see Figure 1.11, section 1.4.4 Albuquerque et al. (2009)).

The reinstatement sessions in this study were 2 hours long. MLA is detected in peak levels in the plasma after 30 minutes of oral and i.v. administration (Turek et al., 1995, Nirogi et al., 2012). Intraperitoneal administration of MLA resulted in increasing brain levels of MLA reaching a peak at 30 minutes post-injection and declining until approximately 90 minutes post-injection (Turek et al., 1995). The testing time is therefore sufficient for the potential detection of changes in behaviour induced by MLA, especially as reinstatement responses occurred in the first hour of testing. There are currently no time-dependent studies on brain levels of MLA following subcutaneous administration, therefore comparison to the results in this thesis is difficult, but the route and dose of MLA is worth considering for future studies.

A behavioural side effect of using MLA appears to be novel object memory impairment, shown by the impairment of novel object memory recognition 24 hours after systemic administration of MLA (Tinsley et al., 2011). This study used surprisingly low concentrations of MLA (87.5µg/kg, i.p.) and found an impairment of novel object recognition 24 hours after systemic administration, but not 20 minutes post-injection. Infusions of MLA into the mPFC resulted in the inhibition of the encoding, but not retrieval of associative recognition memory in rats, whereas infusion of DHβE resulted in the inhibition of retrieval but not encoding of these memories, demonstrating a subtype-specific differential role of nAChRs in associative recognition memory. Infusions of MLA into the PFC, however had no

effect on the morphine-primed reinstatement in rats (Wright et al., 2018), suggesting different networks involved in associative memory recognition and reinstatement of CPP.

With additional time, the heroin self-administration protocol with the considerations discussed above could be improved to enhance lever responses. It would be interesting to investigate the effect of MLA on heroin-primed reinstatement and cue-primed reinstatement of drug seeking individually, and to study the effects of different doses of MLA. Wright et al. (2018) showed that i.c.v. infusions of MLA in the ventral hippocampus inhibited morphine-primed reinstatement of CPP in rats. It could be interesting to extend these findings in the heroin self-administration model, by administering MLA locally, rather than systemically prior to IVSA reinstatement.

3.5.5. Differences between IVSA and CPP

Learning and memory processes play a critical role in the development and maintenance of drug seeking behaviour. There are two types of addiction-related learning models: instrumental learning, where the animal learns there is an action-outcome relationship between drug taking and the rewarding experience. The other is Pavlovian learning, where, with repeated drug experience, the animal associates the rewarding effects of the drug with a cue present at the time of drug taking (for review, see Perry et al. (2014)). CPP is a Pavlovian model of learning, which is more passive, where the occurrence of the reward does not depend on the action of the animal. IVSA is an instrumental model of learning and is a more active learning model, where the action of the animal dictates the reception of the

reward. Similar effects are mostly seen in the reinstatement of these two models (for review, see Aguilar et al. (2009)), suggesting the neurobiological basis of these two models of reinstatement generally overlap, however, the distinctions in the model could explain the disparities in the effects of MLA in the CPP and IVSA experiments in this study.

A review by Aguilar et al. (2009) highlighted differences in effects of drug manipulations on either the reinstatement of CPP or self-administration with the same drug of abuse. This effect is especially noted in the case of cross-reinstatement, where priming with other drugs of abuse can induce the reinstatement of CPP. Nicotine, ethanol and morphine all caused the reinstatement of cocaine CPP, whereas none induced the reinstatement of cocaine self-administration. Stressors such as food deprivation and spontaneous withdrawal had no effect on the reinstatement of morphine CPP, whereas they both reinstated heroin self-administration. Conversely, restraint stress induced the reinstatement of heroin self-administration, whereas it had no effect on the reinstatement of morphine CPP (Aguilar et al., 2009). Furthermore, dopamine receptor antagonists have been shown to inhibit the reinstatement of heroin self-administration in rats (Ettenberg et al., 1996, Shaham and Stewart, 1996), whereas they seem to have no effect on morphine CPP in mice (Ribeiro Do Couto et al., 2005), suggesting neurobiological differences in CPP and IVSA. The data from this chapter, combined with other findings in previous literature could point to a distinct and specific role of α7nAChRs in modulating a specific drug-related behaviour which is modelled more closely by CPP.

The methodological differences between CPP and IVSA could help to understand the differences in results observed in this thesis. Firstly, the acquisition of either

behaviour is very different. In CPP, rats received a total of two heroin injections during conditioning, whereas rats underwent 15-20 heroin IVSA sessions, with rats taking up to 20 infusions of heroin per session. Although the heroin administration is contingent with the presentation of cues in IVSA, the chronic administration of heroin may be reflected by differences in brain plasticity compared to CPP, which may reflect more acute changes on plasticity. Furthermore, CPP is a more passive model of drug-paired associations as there is no action required to display acquisition of the behaviour, except spending more time in the drug-paired compartment; whereas in IVSA rats have to actively press a lever on a tough reinforcement schedule (FR5) to obtain the reward. During extinction in IVSA, the rats still have to actively work for saline infusions until they give up, and there is no equivalent for this in CPP. The distinctions of a passive versus active model likely contribute to the differences in effects of MLA on either model obtained in Chapters 2 and 3.

It is possible that CPP and IVSA are correlates for distinct addiction-related behaviours. CPP likely models drug-paired associations and drug preference (i.e. preference for a drug-paired compartment), which may not include psychological dependence on the drug (i.e. craving). Because of the greater exposure to the drug and the requirement of animals to work to obtain the drug, IVSA may model psychological dependence on the drug, and blocking their motivation to drug seeking will be more challenging.

3.6. Conclusions

The results in this chapter showed that:

1) MLA has no effect on the reinforcement of heroin self-administration
2) MLA had no effect on the re-acquisition of heroin self-administration
3) MLA had no significant effect on the heroin-and cue-primed reinstatement of drug seeking.

These results suggest that α7nAChRs do not play a role in mediating the reinforcing effects of heroin, which extends previous findings (Liu, 2014, Wright et al., 2018). The role of α7nAChRs in the reinstatement of drug seeking in the IVSA model is not as clear as in the CPP model. Due to the wide variability in responses in this chapter, a larger study with a greater sample size is needed to elucidate any potential effects of MLA further.

CHAPTER 4 GENERAL DISCUSSION

The results described in this thesis have shown that the α7nAChR antagonist MLA has no effect on the acquisition of heroin CPP, or on either the re-acquisition or breakpoint of heroin intravenous self-administration; but did block the reinstatement of heroin CPP supporting a role for α7nAChRs in retrieval of drug-associated memories. The effects of antagonising α7nAChRs on the heroin- and cue-primed reinstatement of IVSA are however still unclear, suggesting potential differences between CPP and IVSA as correlates of human relapse. Attempts were made to determine the neuronal mechanism by which MLA inhibits reinstatement of opioid CPP, focussing on AMPA receptor expression, but results were inconclusive.

4.1. Putative model for the reinstatement of drug-seeking behaviour

Figure 4.1 describes the putative model for synaptic plasticity mechanisms occurring during the stages of CPP. During the acquisition of heroin CPP, morphine, a metabolite of heroin acts at μ opioid receptors on GABAergic neurons in the VTA, inhibiting GABA release. This leads to the disinhibition of dopamine cells and results in an increase in dopamine release in the NAc, which mediates the rewarding effects of the drug (Hyman et al., 2006). Repeated administration of opioids leads to long-lasting changes within the reward circuitry, including excitatory neurotransmission (Luscher and Malenka, 2011). Glutamatergic, cholinergic and GABAergic pathways interconnect to modulate synaptic strengthening to induce long-term potentiation and increase AMPA receptor responsiveness to glutamate (Kauer and Malenka, 2007). During extinction

training, further changes occur, with the disruption of LTP (Portugal et al., 2014) and the formation of an alternate neutral memory. The drug-related memory is thus suppressed and the conditioned behaviour is extinguished (Bouton, 2002). During reinstatement, the exposure to a priming dose of heroin causes further glutamatergic and dopaminergic signalling, leading to plasticity changes that restore LTP (Moron et al., 2007, Portugal et al., 2014). The drug-associated memory is thus re-activated and this triggers reinstatement.

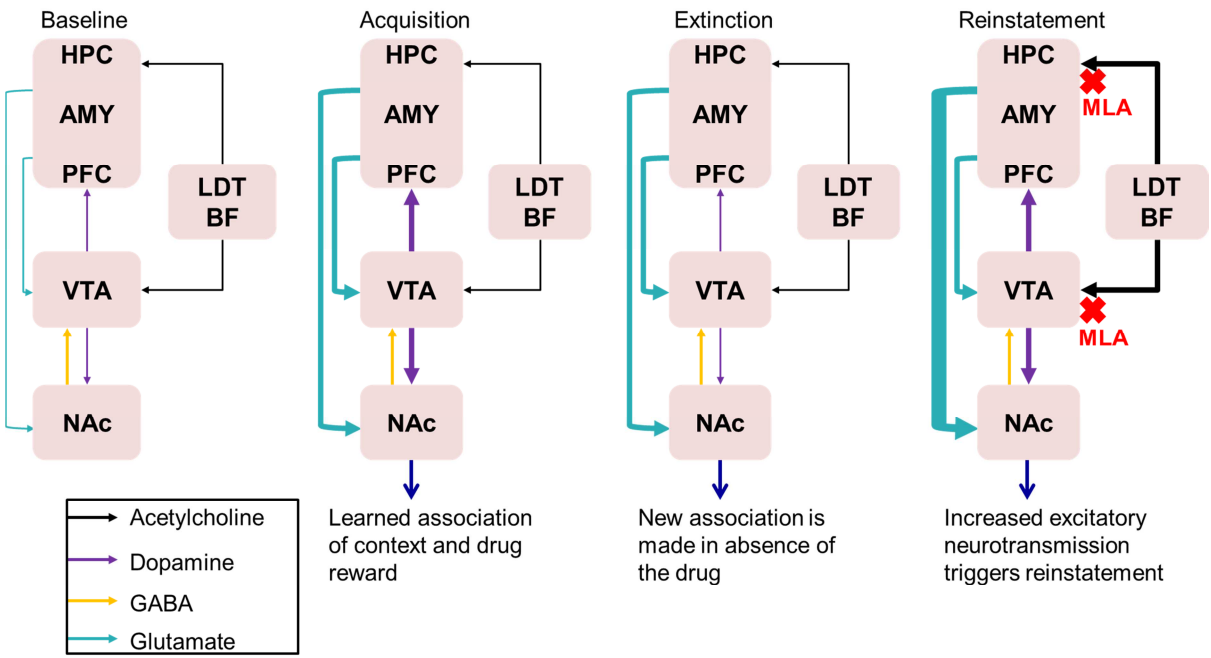


Figure 4.1: Putative model for the acquisition, extinction and reinstatement of drug-seeking behaviour and the effect of MLA on reinstatement. At baseline, dopamine and acetylcholine neurons fire tonically, maintaining basal tone. Acute administration of heroin causes dopamine neurons to switch to burst firing leading to increased levels of dopamine in the NAc, which mediate the rewarding effects of the drug. During acquisition, repeated contextual administration of heroin results in plastic changes in the reward circuitry by the strengthening of glutamate signalling. During extinction, the context-associated memory is rewritten in the absence of the drug and leads to synaptic weakening, or the disruption of LTP. During reinstatement, exposure to a priming dose of heroin causes an increase in dopamine and glutamate firing leading to plasticity changes that restore LTP. The drug-associated memory is thus re-activated and this triggers reinstatement. MLA (red) is proposed to block α7nAChRs in the ventral hippocampus and VTA, which inhibits the excitatory and inhibitory signalling required for LTP, inhibiting the recall of the drug-associated memory, and thus preventing the expression of the conditioned behaviour.

The location of the hippocampus and its interconnectivity with the reward circuitry and cholinergic innervation places it in an ideal position to influence synaptic plasticity events occurring during the different stages of addictive behaviours (Woolf and Butcher, 2011, Kutlu and Gould, 2016). Importantly, it appears that hippocampal theta oscillations are considered particularly important in learning the significance of drug-associated stimuli, and α7nAChRs can alter these oscillations (Apartis et al., 1998, Cheng and Yakel, 2015, Hernandez-Perez et al., 2016, Robinson et al., 2016). Based on the results from Chapter 2 and the findings by Wright et al. (2018), it is proposed that opioid priming induces increases in diffuse acetylcholine in the hippocampus (and the VTA). This increase in ACh levels could activate α7nAChRs, which increases presynaptic intracellular calcium levels, leading to an increase in glutamate release from presynaptic terminals and the enhancement of postsynaptic excitability (Gray et al., 1996, Feduccia et al., 2012). This could favour the induction of alterations in synaptic plasticity, which is believed essential in the manifestation of reinstatement (Shen et al., 2011, Portugal et al., 2014) In-vivo measurements of field EPSPs after heroin-primed reinstatement of self-administration in rats have shown that heroin priming induces NMDA-dependent LTP in the NAc, concurrent with an increase in the diameter and density of medium spiny neuron spines. Furthermore, the inhibition of GluN2B-containing NMDA receptors by the direct administration of either an antagonist or siRNA into the NAc inhibited heroin-primed reinstatement (Shen et al., 2011).Furthermore, CPP studies have shown that, while the acquisition of morphine CPP is associated with enhanced basal synaptic transmission but impaired LTP in the mouse hippocampus, morphine-primed reinstatement of CPP results in a robust enhancement of LTP, which is blocked by an GluN2B-selective

antagonist administered directly in the hippocampus (Portugal et al., 2014). This evidence is compelling as both studies show a strong involvement of NMDA-dependent LTP in the reinstatement of opioid seeking in brain regions known to be involved in modulating drug-seeking behaviours.

In Chapter 3, there was no significant effect of MLA on the cue- and drug-primed reinstatement of IVSA, which conflicted with the CPP results of Chapter 2. This prompts reflection on the behavioural and neurochemical differences that govern reward-related learning paradigms. As mentioned previously, numerous brain regions contribute to the development and expression of addiction-related behaviours. It is possible that IVSA involves other brain regions such as BNST, ventral pallidum and lateral habenula, to a greater extent than CPP (Brown et al., 2010). These brain regions have yet to be shown to be modulated by α7nAChRs.and as such; antagonism of α7nAChrs in the ventral hippocampus would be insufficient to block IVSA.

The major difference between CPP and intravenous self-administration is that the former is a classical Pavlovian model of drug-related learning, whereas the latter is an operant model. Both of these paradigms are used to study addiction-related behaviours, and the reinstatement stage of these paradigms appears to have a certain predictive validity as conditions that trigger craving and relapse (drug re-exposure, contextual cues and stress) in humans also reinstate drug seeking in animals.

Treatments for addiction to drugs of abuse have come from preclinical studies and either reduce withdrawal symptoms, substitute for or block the reinforcing effects of the drug of abuse (Bart, 2012). Reviews of these treatments however show that

in the long term, they may not prevent relapse (Dong et al., 2017), which sheds light on another limitation of current behavioural paradigms, which have short time frames. Studies have shown that to a certain extent, there are distinct and additional brain regions that mediate cue- and drug-induced reinstatement compared to drug reinforcement and abstinence (Stewart, 2008, Rogers et al., 2008, Namba et al., 2018). In reinstatement studies, the majority of compounds that have an effect on cue-induced reinstatement of self-administration have the same effect in drug-primed reinstatement (Perry et al., 2014). There are however exceptions which highlight potential differences in the brain regions involved in the reinstatement in response to either stimulus, especially in studies with opioids. For example, D1 and D2 receptor antagonists appear to have no effect on stress-primed reinstatement of heroin seeking, whereas they inhibit drug-primed reinstatement (Shaham and Stewart, 1996). Additionally, an OX1 orexin receptor antagonist decreased the cue-primed reinstatement of heroin seeking but had no effect on drug-primed reinstatement (Smith and Aston-Jones, 2012). A limitation of studies of potential treatments on the reinstatement of opioid self-administration however is that the effects of the drug are rarely studied on the reinstatement in response to both stimuli in the same study. Further research into the potential differences in contributions of brain regions is therefore needed.

There are also instances of pharmacological manipulation of the reinstatement of CPP and IVSA studies which contradict each other, bringing into question whether CPP and IVSA are isomorphic paradigms. The main argument of this is the contradictory evidence of dopaminergic transmission in the reinstatement of CPP or IVSA. As previously discussed in Chapter 1 (Section 1.4.4.1.), many studies have shown that antagonising dopamine receptors (D1 and D2) inhibited the

drug-primed reinstatement of heroin IVSA (Shaham and Stewart, 1996, See, 2009); whereas they appear to have no effect on morphine-primed reinstatement of CPP (Ribeiro Do Couto et al., 2005), and lesion studies in CPP suggest a potential role of dopaminergic transmission, however methodological considerations limit these findings (Wang et al., 2002). Aguilar et al. (2009) have summarised many other differences in findings in the reinstatement of either CPP or IVSA with opioids and cocaine and have shown that stressors (such as restraint stress or food deprivation) which induce the reinstatement of morphine or cocaine CPP do not all reinstate heroin or cocaine IVSA, cross-reinstatement of opioid CPP and IVSA with other drugs of abuse produce different responses, and manipulations of the corticotrophin system (for opioid drugs) and glutamatergic transmission (for cocaine) also have opposing effects in both behavioural paradigms.

The advantage of CPP is that it is a relatively inexpensive and simple model for measuring reward-based learning. On the basis of detecting drug reinforcement, both CPP and IVSA can be argued to be overlapping techniques, as drugs such as cocaine, heroin and methamphetamine that produce CPP produce self-administration. Conversely, drugs that have been shown not to produce CPP (such as dopamine, opioid and acetylcholine receptor antagonists or antidepressants) also do not produce self-administration (Bardo and Bevins, 2000). There are however some instances of drugs that produce either CPP or self-administration and do not produce a response in the other method (LSD, buspirone, PCP, pentobarbital Bardo and Bevins (2000)).

4.2. How do CPP and IVSA relate to human relapse?

Although both CPP and IVSA are well established methods with a certain predictive validity for reward-related behaviours, they are more likely correlates of a specific type of behaviour which is observed in addicts and not of addiction as a whole. Addiction is defined as a chronically relapsing disorder consisting of compulsive drug seeking and taking despite negative consequences. In CPP, the drug-paired association is made with very few drug administrations, so this occurs in the absence of a compulsive drug-seeking state. In IVSA, animals voluntarily self-administer drugs, but there is no negative consequence to taking the drug. Would these behaviours be different in the presence of a negative consequence? This could be investigated by introducing a mild punishment element to the paradigm, where animals would receive a mild punishment paired with each infusion (in the case of IVSA) or in paired with each entry to the drug-paired side (in the case of CPP), and the animal would have a choice to seek the reward despite the punishment (Vanderschuren and Everitt, 2004, Pelloux et al., 2007). Theoretically this could model the compulsive need to take drugs despite negative consequences seen in humans. An alternative method could also be by devaluing the reinforcer by pairing it with an aversive drug. Concurrent injections of lithium with food has been shown to decrease responding for food, but not when it is paired with cocaine or ethanol, indicating habitual use over the drug's incentive value (Panlilio and Goldberg, 2007). The escalation of use is another aspect of addiction for some drugs that is not seen in a fixed ratio paradigm of IVSA or CPP. In the case of CPP, this is difficult to model. In IVSA studies, animals given extended access to drugs have been shown to increase their rates of drug intake over time, related to a loss of control over intake (Bozarth and Wise, 1985).

Furthermore, it has repeatedly been shown that reinstatement is more likely in rats that have been given extended access to drugs. There are ethical limitations to this method as rats and primates will neglect grooming, feeding or will overdose (Bozarth and Wise, 1985).

4.3. α7nAChRs as a clinical therapeutic target

This work and previous studies (Feng et al., 2011, Liu, 2014, Wright et al., 2018) have begun to reveal the α7nAChR as an interesting target for the treatment of addiction to substances of abuse (nicotine and opioids so far). What makes it a particularly interesting target is that is seems to specifically modulate relapsing behaviour, which is a major failure of current treatments. The CPP results suggest that MLA inhibits either the drug or cue-primed association of the drug and the context, which is a well reported trigger for cravings. The IVSA results from this thesis suggest that antagonising α7nAChRs may be effective in blocking the triggering of cravings by passive cues, but perhaps it is not effective in blocking the more active aspect of taking drugs and relapsing. It is unlikely that antagonising α7nAChRs is sufficient to treat addiction when given alone, but potentially as an adjunct to current therapies which prevent the negative affective states of withdrawal and block the reinforcing effects of the drug of abuse. The premise of treatment would be that it should be taken prior to being exposed to a drug-associated stimulus; therefore the timing of treatment is also crucial. We have not yet shown whether blocking α7nAChRs following the exposure to a cue would still prevent reinstatement, which would correlate for an addict being exposed to a drug-paired stimulus and taking treatment at that time. If a compound had rapid absorption rates, then this could be taken as a preventative method for

when a patient is about to enter a high-risk situation (e.g. An environment associated with prior drug use) or taken ‘on demand’ when a patient’s craving level increases. This as-needed approach to treating addiction has been used for treating alcohol addiction with the opioid receptor antagonist nalmefene, which has been shown to significantly reduce drinking (Francois et al., 2015), however the methodology of clinical studies has brought the significance of their results into question (Fitzgerald et al., 2016).

Although α7nAChR antagonists might work as on-demand therapies, it is unlikely that they would be clinically relevant as chronic treatments, as α7nAChRs are present in many brain regions and involved in a variety of cognitive behaviours, and putative treatments targeting α7nAChRs for cognitive disorders such as schizophrenia and Alzheimer’s disease, are agonists (Albuquerque et al., 2009). Antagonising α7nAChRs may therefore produce negative cognitive and emotional states in patients that would result in the discontinuation of treatment. A better route of therapeutic investigation might be to explore negative allosteric modulators (NAMs). This approach would allow the down-regulation of α7nAChR activity, whilst still allowing basal endogenous function thereby reducing aversive side effects. An interesting endogenous NAM is kynurenic acid, which has been proposed to be a NAM of α7nAChRs (Albuquerque and Schwarcz, 2013), and has been reported to inhibit the reinstatement of nicotine, cocaine and cannabinoid IVSA in squirrel monkeys (Justinova et al., 2013, Secci et al., 2017). Kynurenic acid is also an antagonist of the three classes of ionotropic glutamate receptor, AMPA, NMDA and kainate, with a similar affinity for NMDA receptors as α7nAChRs. To a certain extent, kynurenic acid has benefits as a glutamate receptor antagonist due to its neuroprotective and anticonvulsant effects; however

the non-selective inhibition of excitatory currents in the brain is bound to have a multitude of undesirable effects. Furthermore, the affinity of kynurenic acid for the α7nAChR is still unclear, ranging from 10 to 100μM (Albuquerque and Schwarcz, 2013), depending on the methodology used (cell cultures or brain slices, method of drug application, age of animal, native or heterologous receptor expression etc.), therefore further work is needed to characterise kynurenic acid as an α7nAChR NAM. Nonetheless, future compounds could be developed based on the structure of kynurenic acid but with improved affinity for α7nAChR over glutamate receptors. Multiple α7nAChR positive allosteric modulators (PAMs) are currently being investigated as potential treatments for cognitive diseases (Yang et al., 2017), and the structure-activity relationship can be studied to identify residues that govern positive and negative modulation. Recently, Gill-Thind et al. (2015) have developed α7nAChR ligands differing only in methyl substitutions on a single aromatic ring, which greatly alters their pharmacology and identified 5 different subclasses of α7nAChR ligand, including PAMs and NAMs. In addition, the recent improvement of the acetylcholine binding protein model by introducing further humanising mutations will allow accurate studies of the crystal structure of α7nAChR allosteric binding sites for rational drug design (Delbart et al., 2018). These are promising avenues for drug discovery and potential therapeutics.

This rate of absorption into the brain would be a factor of the dose, the route of administration and the pharmacokinetics of the compound, all of which need to be considered to produce an effective anti-relapse therapy. MLA has been shown to have a short elimination half-life following i.v. administration (approx. 1.3 hours) and a low bioavailability following oral administration (approx. 17%) (Turek et al., 1995, Nirogi et al., 2012). Peak plasma levels of MLA were reached at 30 minutes

following both i.v. and oral administration (Nirogi et al., 2012). In addition regional brain distribution in rodent brains showed that MLA was rapidly taken up into the brain (approx. 10 mins after i.v. administration). For the facilitation of daily treatment, the bioavailability of an α7nAChR ligand would need to be improved. However, as MLA enters the bloodstream easily, There appears to currently be no literature on the pharmacokinetics of MLA in humans.

The route of administration would depend on the compound, but a rapid delivery to the brain would be needed to abolish arising cravings. The route of administration of a potential therapy would depend on whether it would be used chronically or on demand. For example, varenicline is used chronically; therefore it needs to reach a steady state but does not need a fast onset of action. Dermal patches and oral administration are slow routes of administration which achieve a steady state in the patient and do not need a fast onset of action; therefore they would be better for chronic use. For an ‘on-demand’ treatment, faster routes of administration are required to rapidly prevent craving symptoms. Inhalers, nasal sprays and buccal delivery (under the tongue) are rapid methods of drug delivery for high risk situations. This would depend on the properties of the ligand, but this type of route of administration would potentially produce more rapid delivery than oral consumption. The advantage of an α7nAChR NAM is that it is likely to have little abuse liability: nicotine is an agonist, and primarily targets heteromeric nAChRs to produce dependence as the more desirable effects come with agonism of the receptor.

CHAPTER 5 CONCLUSIONS AND FUTURE WORK

5.1. Conclusions

The aim of this thesis was to examine the role of α7nAChRs in motivational memory in the context of drug reward using conditioned place preference and intravenous self-administration (IVSA). The major findings and conclusions are below:

- Systemic pre-treatment with the selective α7nAChR antagonist methyllycaconitine (MLA) results in the selective inhibition of the reinstatement of heroin CPP in rats.
- The effect of MLA on the drug- and cue-primed reinstatement of heroin IVSA is still undetermined.
- MLA has no effect on the motivational value of heroin, demonstrated in both CPP and IVSA paradigms.
- The synaptic plasticity events occurring during reinstatement and the effect of MLA on plasticity are still undetermined.

The results from this thesis corroborate the previous evidence of a role of α7nAChRs in modulating the reinstatement of CPP selectively, and extend their findings with a more rewarding and clinically-relevant opioid, heroin. The aim was also to extend these findings in the IVSA model, but due to the study being underpowered, further work is needed to elucidate the role of α7nAChRs in the reinstatement of this behavioural paradigm. Further work is also needed to determine the synaptic plasticity events which occur at the different stages of CPP and IVSA, and to elucidate the role of endogenous acetylcholine in modulating relapse-like behaviour in these paradigms. Understanding the associative memories that are formed between drugs of abuse and contextual stimuli has crucial implications for treatment, as one of the major failures of current therapies is the lack of prevention of relapse.

5.2. Future Work

5.2.1. Improving the behavioural paradigms

As previously discussed, while both the CPP and IVSA paradigms are well-established correlates for specific types of addiction-related behaviour, they are not complete models of addiction. To improve these models, the protocol could include traits more relevant to humans, such as an element of seeking reward despite negative consequences (foot-shock punishment), extended access to drugs, voluntary abstinence during extinction and potentially an element of choice during reinstatement.

The main issue with the results in Chapter 3 (IVSA) was that the study was underpowered. The study would need to be repeated with additional subjects in each treatment group. Furthermore, as previously mentioned, although there are overlapping brain regions involved in drug- and cue-primed reinstatement, there are also distinct regions which are activated in response to either stimulus. In this study, reinstatement was achieved by exposure of drug **AND** cue. The next aim would be to repeat this study but expose the animals to drug **OR** cue to observe any potential effect of MLA on either cue- or drug-primed reinstatement of self-administration. Additionally, MLA could be delivered intracranially, as this was shown to have a more pronounced effect on reinstatement of morphine CPP when administered directly into the ventral hippocampus than systemically (Wright et al., 2018). It is also possible that the dose of MLA needs to be increased for IVSA experiments, as Liu (2014) found a more marked effect on the reinstatement of nicotine IVSA with 10 mg/kg MLA.

5.2.2. Investigating the role of cholinergic pathways in reinstatement

To determine a temporal role of cholinergic signalling, levels of diffuse acetylcholine could be measured in regions of interest during reinstatement. Regular microdialysis experiments would not allow real-time measurements of endogenous acetylcholine levels in brain regions, however, there is a technique for in-vivo measurements of ACh with a sub-second resolution (Sarter and Kim, 2015). This method would allow the time-dependent changes in cholinergic transmission during the different stages of motivational memory paradigms. This could then be correlated with the in-vivo behaviour to further our understanding of cholinergic transmission in drug seeking behaviour.

The invention of optogenetics and its broad applications has greatly advanced our understanding of neuronal networks underlying behaviour. This method allows the temporal control of genetically defined neurons in live animals by light (Deisseroth et al., 2006). Witten et al. (2010) have shown that the optogenetic activation of cholinergic interneurons in the NAc increases the frequency of inhibitory currents and suppresses medium spiny neuron spiking, and vice versa. This study also showed a role of cholinergic interneurons in the acquisition of cocaine CPP in mice, as the silencing of these interneurons resulted in a decrease in preference for the drug-paired chamber (Witten et al., 2010). These experiments demonstrate that there is potential to use optogenetic manipulation of cholinergic inputs to brain regions to determine their role in addiction-related behaviours, which could also be applied to the IVSA paradigm.

Microdialysis and optogenetics could allow the measurement and manipulation of cholinergic pathways but do not enable the investigation of the role of these pathways in drug-seeking behaviour at a receptor-specific level. The use of other

models and methods could enhance our knowledge of the selective role of α7nAChRs in reinstatement.

5.2.3. Investigating the selective role of α7nAChRs in reinstatement

The use of transgenic animals can be useful to study the selective role of α7nAChRs in reinstatement. It has previously been shown that mice lacking the α7nAChR subunit are still able to acquire nicotine CPP (Walters et al., 2006b) and IVSA (Pons et al., 2008), however it is possible that compensatory mechanisms account for the lack of α7nAChRs. It could be more relevant to use conditional knockouts (Hernandez et al., 2014) to induce deletions specifically in a tissue neuron type. This could allow a targeted and selective investigation into the role of α7nAChRs on dopaminergic glutamatergic or GABAergic signalling in a specific brain region, such as the ventral hippocampus in behavioural paradigms. Temporal control of α7 gene expression by inducing knockouts during later stages of development of the animal could also allow a study of the role of these receptors without confounding results from compensatory mechanisms, though currently, there are no such transgenic animals in existence to our knowledge.

Immunohistochemical quantification of fos expression (acting as a marker of neuronal activation) in neurons could also reveal different brain regions activated during the reinstatement of IVSA and CPP. The effect of MLA on the activation of these brain regions or the investigation of the α7 knockout mice could shed some light on the role of α7nAChRs in regulating the activation of these brain regions. There are also c-fos-GFP transgenic mice and more recently, rats, which have GFP tags transcribed onto fos to identify recently synthesised fos (Barth et al.,

2004, Katoh et al., 2014). These transgenic animals could be used to identify specific brain regions recently activated in response to different stimuli, and to investigate the role of α7nAChRs in regulating the activation of these regions.

5.2.4. Investigating synaptic plasticity events during reinstatement

There are also differences in brain regions involved in the reinstatement of CPP and IVSA. Due to these differences, it could be beneficial to identify the synaptic plasticity events or activation of brain regions during reinstatement, in both behavioural models, and the effect of MLA on these events. This could be done by improving the techniques used in the current study for synaptic AMPA receptor quantification. The current study investigated synaptic plasticity changes occurring immediately after memory recall and focussed on the phosphorylation of GluA1-containing AMPA receptors, however other changes also occur at later stages of LTP to stabilise the synapse. For example, the GluA2 AMPAR subunit also plays a significant role in the regulation of synaptic transmission by regulating receptor kinetics, single channel conductance and calcium permeability (Oh and Derkach, 2005). It is one of the most tightly regulated subunits in terms of gene expression, RNA editing, receptor assembly and trafficking, suggesting it plays a major role in AMPAR structure, function and synaptic transmission. During LTP, calcium-permeable GluA1 homomers are exchanged for Ca^{2+}-impermeable GluA2-containing AMPAR to stabilise the synapse (Isaac et al., 2007). It is therefore important to measure the synaptic expression of this subunit as well as GluA1 in future studies.

Another method of measuring synaptic strength is electrophysiology. The role of α7nAChRs in the modulation of synaptic plasticity is well documented and previous work in this lab has shown that MLA inhibits LTP in the mPFC (Udakis et al., 2016); and this has also been reported in the hippocampus (Cheng and Yakel, 2015). Further electrophysiological experiments could be used to elucidate changes in LTP in the ventral hippocampus after the reinstatement of either CPP or IVSA, and the effects of MLA on LTP. In addition, improvements in in-vivo electrophysiological methods could allow real-time recordings of synaptic transmission occurring during the behavioural paradigm. Transgenic α7 knockout mice (mentioned below) could also be used in these experiments to determine their effect on signalling in these behavioural paradigms.

5.2.5. Clinical relevance

As previously discussed (Chapter 4, section 4.3), the use of an antagonist is not clinically relevant due to undesirable generalised effects. A more relevant ligand for α7nAChRs would be a negative allosteric modulator. To progress the findings in this thesis further, it would be interesting to investigate the effects of an α7nAChR NAM on the reinstatement of heroin CPP. As there are few effective α7nAChR NAMs currently in existence, much work is initially needed in the development of such a compound. As previously discussed, some novel α7nAChR NAMs have been developed with structural homology to a previously discovered allosteric agonist (Gill-Thind et al., 2015). Perhaps one of these compounds could be an interesting ligand for preclinical studies, however it still remains to be determined if the allosteric site is the same as that characterised by Neill Millar. A

concern of using a NAM however is that its effect on reinstatement will be more dilute and potentially non-existent, as receptors will retain basal levels of function.

The present study has confirmed that MLA selectively inhibits the reinstatement of heroin CPP, which corroborates previous findings with morphine (Feng et al., 2011, Wright et al., 2018). It seems this effect is therefore generalised to opioids, and drugs of abuse share common neurobiological mechanisms to a certain extent, therefore we could hypothesise that MLA may inhibit the reinstatement of other classes of drugs of abuse. There is already some evidence of nicotinic receptor (and even α7) involvement in addiction processes for other drugs of abuse, such as alcohol, cocaine, cannabinoids, ethanol (Rahman et al., 2015) and methamphetamine (conflicting evidence (Verrico et al., 2014, Pittenger et al., 2017)), though there is still much to be investigated.

These future experiments would extend the findings from the current study in terms of the role of α7nAChRs in the reinstatement of drug seeking in behavioural paradigms. Additionally, these experiments could allow us to develop and improve our understanding of the behavioural models used as correlates of addictive behaviours.

AN INTRODUCTION TO THE APPENDICES

Appendix A will show the optimisation of subcellular fractionation and Western blot experiments to quantify AMPA receptor changes in the postsynaptic membranes in the hippocampi of mice that underwent morphine-primed CPP. Appendix B will expand on the novel use of near-infrared scanning technology for immunohistochemistry, alongside confocal microscopy as a novel method of synaptic protein quantification. These different methods aim to extend autoradiography data found in our lab further to identify subunit-specific changes in AMPA receptor expression at the postsynaptic membrane.

APPENDIX A METHODS OF AMPA RECEPTOR QUANTIFICATION

A.1. Introduction

As previously discussed, Wright et al. (2018) found a significant increase in [^{3}H]-AMPA binding in the ventral hippocampus of mice that underwent morphine-primed reinstatement of CPP. This increase in [^{3}H]-AMPA binding was blocked by MLA administration prior to morphine-primed reinstatement, suggesting a modulatory role of α7nAChRs on glutamatergic signalling in the ventral hippocampus during reinstatement. Autoradiography however only measures the total number of receptors bound, regardless of subcellular location or the subunit composition of these receptors. To elucidate changes occurring at a synaptic level, subcellular fractionation accompanied by Western blot quantification was selected to isolate postsynaptic membranes.

Subcellular fractionation is a method of separating organelles, such as nuclei, mitochondria, plasma membranes and ribosomes, based on their density. It is ideal for the enrichment and isolation of low abundance proteins in specific cellular compartments. The fractionation process involves the disruption of the cellular organisation and the fractionation of cellular compartments using differential centrifugation (Lassek et al., 2015). The methodology used in this study was based on methods used by Billa et al. (2009) and Portugal et al. (2014), who isolated postsynaptic membrane fractions using density gradient centrifugation to show changes in synaptic plasticity during different stages of morphine CPP (in mice and rats). This technique would allow the isolation of postsynaptic membranes from whole brain region homogenates for the quantification of proteins inserted in the postsynaptic membrane. NMDA-dependent LTP is characterised by the insertion of AMPA receptors in the postsynaptic membrane (Luscher and Malenka, 2012), and studies have demonstrated synaptic plasticity alterations in

CPP (Ma et al., 2007, Billa et al., 2009, Portugal et al., 2014). Subcellular fractionation would therefore allow the quantification of AMPA receptors only at postsynaptic membranes, excluding extrasynaptic and intracellular receptors stores, to characterise changes in synaptic plasticity in morphine CPP. The aim of this study would then be to apply this technique to investigate the role of α7nAChRs in modulating these changes in synaptic plasticity.

A.2. Materials and methodology

Animals

Initial optimisation of the subcellular fractionation and Western blot experiments was performed using tissue taken from naïve male C57/BL6 mouse brains (6-9 weeks). Thereafter, hippocampi were dissected from animals (C57/BL6 mice (8-10 weeks old)) that had previously undergone reinstatement of morphine-induced CPP.

Subcellular Fractionation

Subcellular fractionation methods were based on previous publications by Billa et al. (2009) and Portugal et al. (2014). The steps of the protocol are shown in Figure A.1.

Mouse hippocampi (whole hippocampi, right and left side pooled) were processed following the methodology by Portugal et al. (2014). Hippocampi were homogenised on ice using a glass homogeniser in 1.5 mL homogenisation buffer containing 0.32 M sucrose (to maintain osmotic pressure), 0.1 mM CaCl2, 1 mM

MgCl2 and 1 mM phenylmethylsulfonyl fluoride (PMSF, protease inhibitor). Approximately 200 µL of hippocampus homogenate were reserved for immunoblotting prior to centrifugation. The remaining homogenate was transferred to 10 mL polycarbonate tubes (Beckman Coulter) and the sucrose concentration was then increased to 1.25 M by adding 1.86 mL 2 M sucrose (with 0.1 mM $CaCl_2$). The homogenate was then overlaid with 1 M sucrose. The less dense 1 M sucrose remains in suspension above the denser 1.25 M sucrose-containing homogenate. Tubes were centrifuged at 100 000 x g for 3 hours at 4℃ (Beckman Coulter Ultima ultracentrifuge). The synaptosome fraction at the 1.25/1 M sucrose concentration interface was collected using a syringe. To isolate synaptic junctions, the synaptosomes were diluted 1:10 in 20 mM Tris-Cl, pH 6 containing 1% TX-100 and mixed at 4℃ for 20 minutes prior to centrifugation at 40 000 x g for 20 minutes. To separate the presynaptic membranes from the postsynaptic membranes, the pelleted synaptic junctions were collected and resuspended in Tris-Cl, pH 8 containing 1% TX-100, mixed for 20 minutes at 4℃ then centrifuged at 40 000 x g for 20 minutes. The postsynaptic membrane fractions (postsynaptic densities, PSD) are insoluble at pH 8, whereas the presynaptic membrane fractions (PAZ) remain soluble. Samples were kept on ice for the duration of experiments or stored at -80℃ until further use.

Protein Concentration Determination

The method used for measurement of protein concentrations was the Bio-Rad DC protein assay, similar to the Lowry assay but improving on the stability of the reagents in the presence of protein and the rapidity of the colour change (Noble and Bailey, 2009). This assay measures proteins in detergent, where other

methods of protein measurements would be inappropriate due to interference with dyes (e.g. Bradford protein assay).

Standard protein concentrations were made up with serial concentrations of BSA (0.025-2 mg/mL) in the same buffer as the samples to measure. A blank sample contained the buffer alone. Reagent A* was made up with 20 µL of reagent S per 1 mL of reagent A. 5 µL of the standards and samples were added to a clear, 96-well plate (Corning), then 25 µL of reagent A* and 200 µL of reagent B and mixed. The samples were incubated for a minimum of 15 minutes before measuring the absorbance in a microplate spectrophotometer at 750 nm. Each standard and sample was measured in duplicate for increased accuracy. The BSA standard curve was used to measure sample protein concentration.

Immunoblotting

Protein samples obtained from subcellular fractionation were boiled at 70℃ for 10 minutes in Laemlli buffer to denature proteins and remove quaternary structures to allow efficient running. Equal amounts of protein (5, 10 or 20 µg) were run along a protein ladder (NEB Colorplus P7709s) at 120 V for 1 hour and 20 minutes in a 7.5% acrylamide gel and electrotransferred onto PVDF membranes at 0.45A for 45 minutes. Some PVDF membranes were cut horizontally between the 80 kD and 58 kD markers to allow blotting for different proteins on the same membrane. Membranes were then blocked with 5% (w/v) BSA in TBST (20 mM Tris, pH7.4, 0.1% Tween 20 (w/v), and 150 mM NaCl) for 1 h at room temperature. Proteins were probed with primary antibodies: rabbit anti-PSD-95 (1:1000, Abcam), chicken anti-syntaxin-1 (1:2000, Sigma) rabbit anti-GluA1 (1:1000, Millipore), rabbit

anti-GluA1 (phosphoSer845, 1:1000, Millipore), chicken anti-βIII tubulin (1:500, Abcam) and incubated overnight at 4℃. Tagged secon dary antibodies used were: donkey anti-rabbit (1:10000, Li-Cor) and donkey anti-chicken (1:10000, Li-Cor). Antibody signals were measured using the Li-Cor Odyssey Clx near-infrared scanner.

Morphine CPP

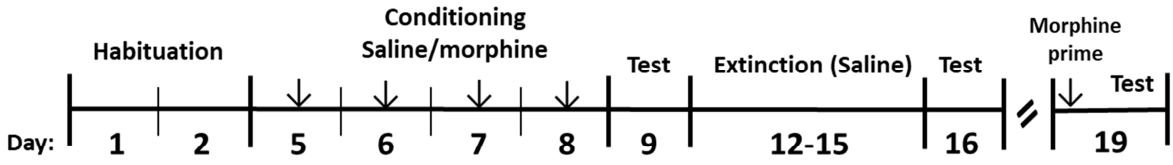


Figure A.1: Mouse morphine CPP injection protocol. Downward arrows ↓ represent saline (10 mL/kg) or morphine (10 mg/kg) injections. The morphine priming dose was 5 mg/kg, i.p. No testing was done on weekends (days 10-11 and 17-18).

Morphine CPP procedures were conducted in the same way as in Chapter 3. The experimental protocol is represented in Figure A.2. The mouse CPP apparatus is shown in Figure A.2. Briefly, all mice were handled one week prior to experimentation. Mice underwent two habituation sessions to establish baseline preference and the means of the sessions were taken as their baseline preference. They were pseudo-randomly assigned a compartment (vertical stripes or black, slightly different apparatus due to the lack of a neutral zone, see **Figure A.2**.1) paired with morphine (10 mg/kg, i.p.) and an unconditioned compartment with saline (10mL/kg, i.p.) on alternate days, over 4 days. On day 9, mice were

recorded free-roaming for 15 minutes in the post-conditioning test. Extinction consisted of 4 days of saline injections in the drug-paired and unpaired compartments on alternate days. The post-extinction test was conducted in a 15 minute session on day 16. On day 19, the mice were either given a saline prime (10 mL/kg, i.p.) or a morphine prime (5 mg/kg, i.p.) and recorded for 30 minutes in the reinstatement test. The first 15 minutes of reinstatement was used for analysis. Results were analysed by one-way ANOVA and Newman-Keuls post-test in GraphPad Prism (version 5).

Mice were then immediately sacrificed by Schedule 1 dislocation of the neck and the dissected hippocampi were snap frozen in ice cold isopentane before being frozen in liquid nitrogen. Hippocampi were stored at -80℃ until further use.

Figure A.2: **Top**: mouse CPP apparatus. The left compartment comprises of black walls with a round-holed floor, whereas the right compartment has vertical stripes and a square-holed floor. Both compartments are separated by a guillotine door, seen open here. **Bottom**: the CPP apparatus placed inside the sound attenuation chamber. Cameras are mounted to the ceiling to record mice inside the boxes.

A.3. Aims

The aim of this study was to identify and optimise an appropriate experiment to detect and quantify changes in postsynaptic AMPA receptor expression or phosphorylation following reinstatement of morphine CPP. Based on previous findings, (Wright et al., 2018), these experiments focussed on the whole hippocampus (due to tissue constraints). Successful experimental optimisation would then allow more detailed examinations into AMPA receptor expression/phosphorylation at different stages of behavioural studies and to quantify changes following pharmacological manipulation of the behaviour.

A.4. Results

Western blot validation in naïve mice

Syntaxin-1 and PSD-95 were used as selective markers for the presynaptic and postsynaptic membranes (Billa et al., 2009). Syntaxin-1 is a protein present in 4 isoforms of approximately 35 kD, part of the SNARE complex involved in docking and fusing vesicles to the plasma membrane for the release of neurotransmitters (Shin, 2011). The antibody selected was selective to membrane-bound syntaxin-1, thus increasing chances of its detection in the presynaptic membrane. PSD-95 is a 95 kD protein belonging to the membrane-associated guanylate kinase (MAGUK) family which acts as a postsynaptic docking protein for larger signalling molecules (de Bartolomeis and Tomasetti, 2012). These two membrane-bound synaptic proteins were selected as positive controls for successful isolation of the pre- (syntaxin-1) and postsynaptic (PSD-95) membranes by subcellular fractionation.

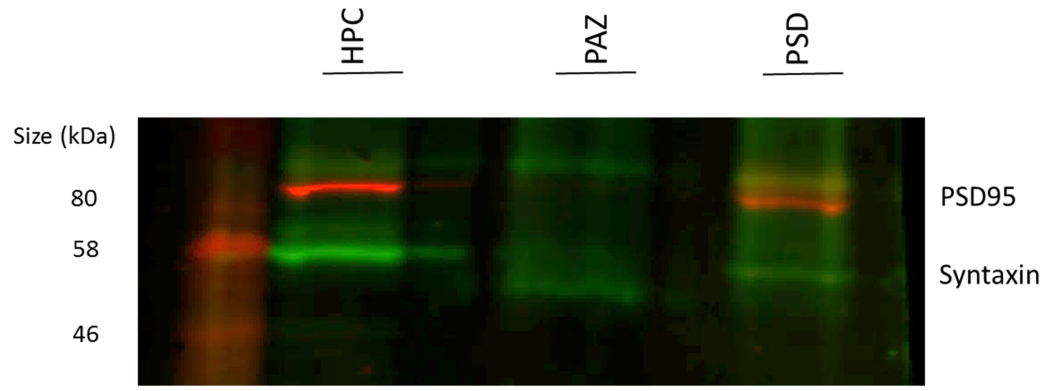


Figure A.3: Western Blot detecting PSD-95 (red) and syntaxin-1 (green) protein bands in rat hippocampus. 20 µg of samples were loaded in each well. HPC: whole hippocampus homogenate, PAZ: presynaptic fraction, PSD: postsynaptic density fraction.

In the unprocessed whole hippocampus homogenate **Error! Reference source not found.**(HPC, **Figure A.3**), both syntaxin-1 and PSD-95 were detected. In the presynaptic fraction (PAZ) only syntaxin-1 was detected, indicating the presence

of the presynaptic marker in this fraction. PSD-95 was present in the postsynaptic fraction (PSD), indicating the presence of the postsynaptic marker in the correct fraction. Syntaxin-1 was however also detected in the PSD fraction, though levels were much lower than in the hippocampus homogenate.

To try and remove presynaptic contamination in the PSD fraction, the PSD sample was resuspended in tris pH 8 and the final centrifugation step of subcellular fractionation was repeated (see methods section). This did not succeed in removing the presynaptic terminals, and Western blot showed the PSD fraction no longer expressed PSD-95 (data not shown).

Overall, these results showed the isolation of postsynaptic membranes (PSD) from a whole hippocampus homogenate, though with some presynaptic membrane contamination. Due to the lack of presynaptic AMPA expression (Billa et al., 2009), it was not deemed necessary to further process the postsynaptic fractions without running the risk of further protein loss.

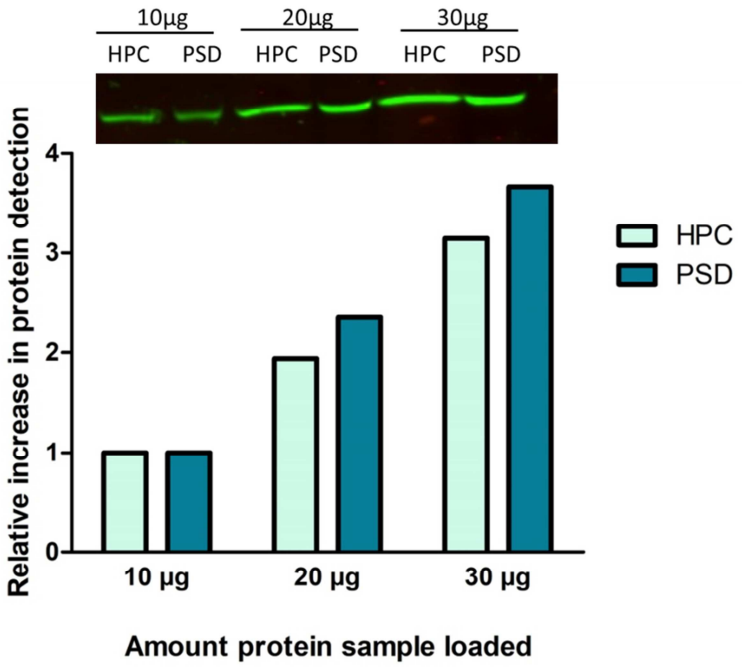


Figure A.4: Relative quantification of βIII-tubulin signals in 10, 20 and 30 µg of whole hippocampal homogenates (HPC) and postsynaptic density (PSD) samples loaded.

βIII-tubulin was selected as the loading control as actin was not detectable in the postsynaptic fractions. βIII-tubulin is a neuronal-specific form of β-tubulin which is anchored to the membrane and therefore should not be washed away during subcellular fractionation. Figure A.4 above shows that βIII-tubulin detection was relatively proportional to the amount of protein sample loaded in both the whole hippocampus homogenate (HPC) and in the PSD fractions (PSD). As the relationship was more linear in the 20µg sample, it was opted to load 20µg of samples for Western blot quantification. Once antibodies against GluA1 and phosphorylated GluA1 (Ser845) were titrated on naïve mouse brain samples, these proteins were probed for in mouse brains that underwent morphine-primed reinstatement of CPP.

Morphine-primed reinstatement of CPP

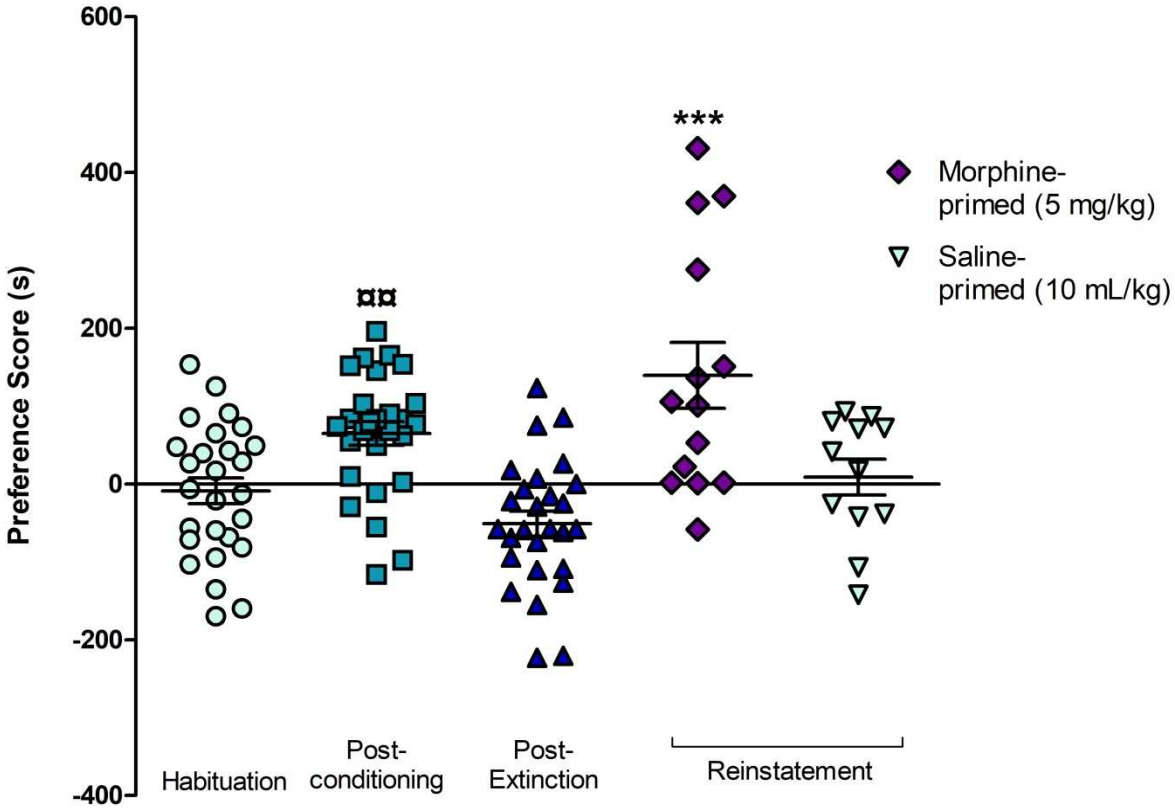


Figure A.5: Morphine-primed reinstatement of CPP. All mice underwent habituation, extinction and morphine conditioning. They were then divided into morphine- (n=14) or saline-primed (n=12) reinstatement groups. One-way ANOVA with Bonferroni post-hoc analysis. Post-conditioning vs Habituation ¤¤p<0.01, morphine-primed reinstatement vs post-extinction ***p<0.001. Data are mean ±SEM.

During habituation, mice showed no preference for the drug-paired side (Figure A.5). In the post-conditioning test, mice showed significant preference for the drug-paired side. This behaviour was then significantly reversed post-extinction. There was no effect of saline priming on preference; however morphine priming significantly increased animals' preference for the drug-paired side, demonstrating the reinstatement of the drug-seeking behaviour. Immediately following reinstatement, mice were sacrificed and their hippocampi were dissected and

processed as described in the methods section above. AMPA receptor subunits GluA1 and phosphorylated GluA1 were probed in postsynaptic membranes to determine an effect of morphine-primed reinstatement on LTP in the hippocampus.

AMPA receptor quantification following reinstatement

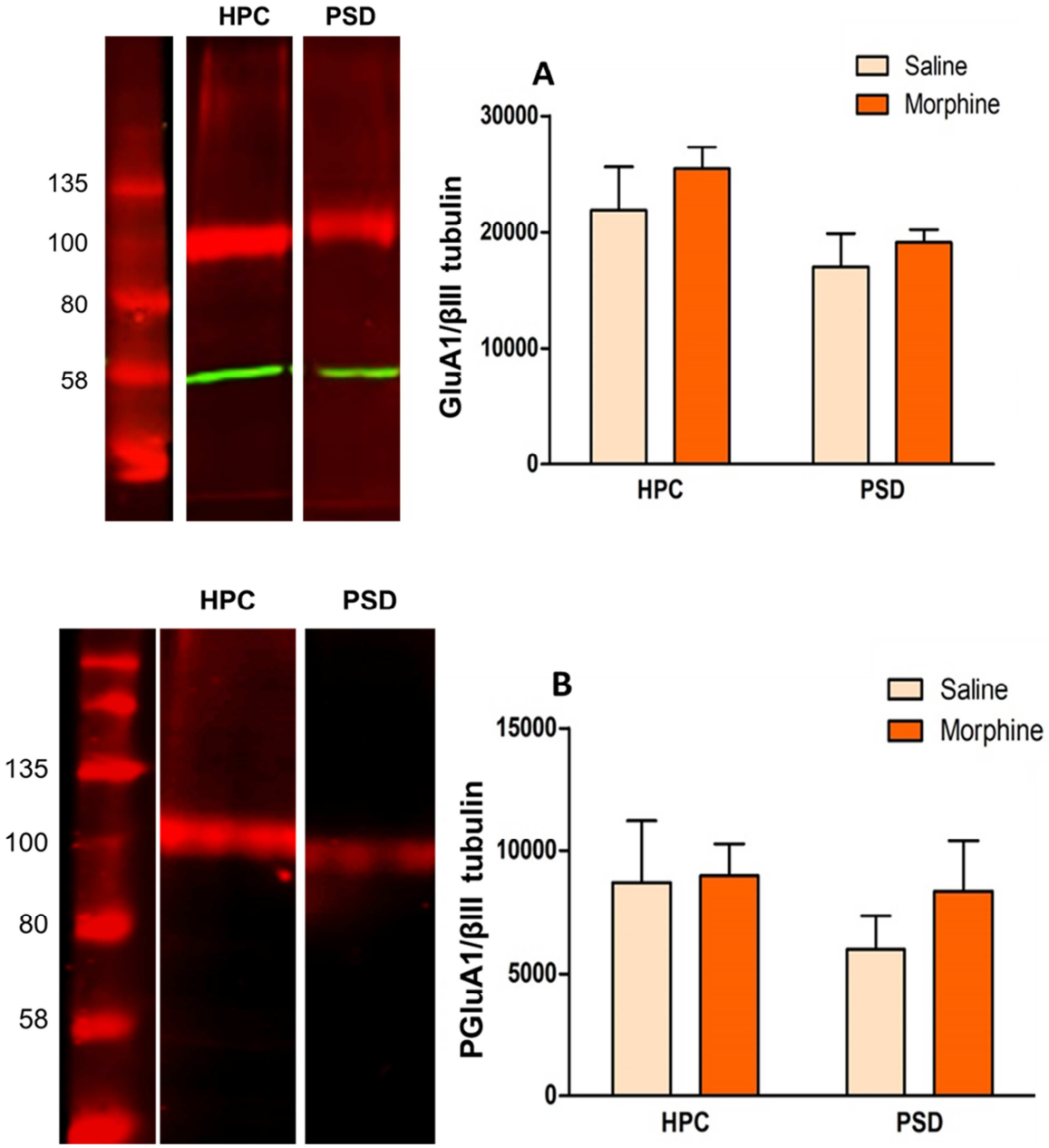


Figure A.6: Effect of morphine or saline-primed reinstatement on GluA1 (**A**) and GluA1 phosphoSer845 (PGluA1, **B**). Signals were normalised to βIII-tubulin in mouse hippocampal homogenates (HPC) and postsynaptic fractions (PSD). Representative blots show GluA1 and phosphor GluA1 in red and βIII tubulin in green. Data are means±SEM. Unpaired t test, not significant, saline: n=5, morphine: n=4.

Western blot quantification (Figure A.6A) showed that there was no difference in the expression of GluA1 subunits between saline and morphine-primed animals in either the whole hippocampus homogenate (HPC) or the postsynaptic membranes (PSD). There seemed to be a slight increase in the expression of GluA1 subunits phosphorylated at serine 845 (Figure A.6B) in the PSD in the morphine-primed animals, though this effect was not significant. Data showing GluA2/3 expression was inconclusive due to the loss of signal in multiple blots resulting in an incomplete data set (only n=2).

These data show that there was no consistent, significant effect of morphine-primed reinstatement on the expression of GluA1 or phosphorylated GluA1 (Ser845) in mouse hippocampi.

A.5. Discussion

Morphine CPP

The mouse morphine CPP data agree with the findings by our and others' many previous studies that these doses of morphine produce significant preference post-conditioning, and induce a significant reinstatement of drug-seeking behaviour ((Do Couto et al., 2003, Feng et al., 2011, Wright et al., 2018); for review, see Bardo et al. (1995)). The aim of this study was to successfully reinstate mice in morphine CPP and to collect the brains for biochemical analysis of postsynaptic AMPA receptor expression by different methods.

Validation of subcellular fractionation and Western blot methodology

This study aimed to optimise and validate the subcellular fractionation methodology used by Billa et al. (2009) and Portugal et al. (2014) to expand on the increases in AMPA receptor binding in the ventral hippocampus triggered by morphine-primed reinstatement of CPP, described by Wright et al. (2018). This method would then be used to quantify postsynaptic AMPA receptor expression at a subunit-specific level.

Quantification of GluA1 and phosphorylated GluA1 at serine 845 at postsynaptic membranes showed no effect of morphine priming compared to saline control. These data do not complement the findings by Wright et al. (2018), nor do they directly contradict them. It is possible that attempting to determine changes at a subunit specific level dilutes any changes in expression seen by total [^{3}H]-AMPA binding by autoradiography. Billa et al. (2009) however found that there was an increase in the phosphorylation of GluA1 subunits at Ser845 during the extinction phase of morphine CPP in rats, therefore demonstrating that this methodology can still be sensitive enough to detect subunit-specific changes in AMPA receptor expression at the postsynaptic membrane. Furthermore, Portugal et al. (2014) found that LTP was enhanced during morphine-primed reinstatement of CPP in mice, which was inhibited by an NMDA receptor antagonist injected directly into the dorsal hippocampus. In this thesis, mouse hippocampi were used, and proteins in individual samples were measured, whereas mouse brain sampled were pooled in the study by (Portugal et al., 2014), which could account for the differences in detection. Overall, the data found in this thesis do not support these previous findings.

Initial validation experiments showed that subcellular fractionation was successful, due to the lack of PSD-95 (the postsynaptic marker protein) in the presynaptic fraction (PAZ). The postsynaptic density (PSD) contained PSD-95 and syntaxin-1, suggesting incomplete separation of pre- and postsynaptic membranes during solubilisation, but as AMPA receptors are not expressed presynaptically (Billa et al., 2009), this was considered negligible. There is evidence showing syntaxin-3 is involved in trafficking AMPA receptors to the postsynaptic terminal by exocytosis from vesicles during LTP (Jurado et al., 2013), so it is possible the anti-syntaxin antibody is non-specifically binding to syntaxin-3 present in the postsynaptic membrane. (Billa et al., 2009) showed no syntaxin-1 in their PSD fractions, though a faint band is visible and these studies used enhanced chemiluminescence (ECL) as their detection method, which is less sensitive to small amounts of protein, especially when detecting larger amounts of protein on the same blot. The near-infrared scanning technology used in this study is more sensitive to small amounts of protein; therefore it is possible there are traces of syntaxin-1 in their PSD fraction which are not detectable by ECL.

The use of βIII-tubulin was deemed acceptable as a loading control due to the proportional increase in detection with the amount of protein loaded and due to it being membrane-bound, there was less risk of protein loss during subcellular fractionation.

Western blot is a well-established technique that has been used to detect the expression of protein in a semi-quantitative manner, by allowing the measurement of protein expression relative to others, but it does not determine the absolute quantity of protein. Using tagged secondary antibodies to detect protein bands has

allowed the better detection of more than one protein on the same blot and improved detection of both faint and strong protein bands.

Overall, it appears that no changes in GluA1 and phosphorylated GluA1 AMPA receptor subunits were detected by subcellular fractionation and Western Blot.

A.6. Conclusions

This study could not replicate the methods used by Billa et al. (2009) and Portugal et al. (2014). The results from this study were inconclusive in quantifying AMPA receptor subunit expression changes in mouse hippocampi which had undergone morphine-primed reinstatement of CPP. Other methods were therefore explored to further investigate the synaptic changes in AMPA receptor expression at a subunit level in the ventral hippocampus. Immunohistochemistry was selected as the method of immunolabelling, as it would more closely resemble autoradiography visualisation in that it would allow region-specific quantification of protein signals in the ventral hippocampus. Two novel methods of quantification were explored and developed: near-infrared whole-slice imaging by Li-Cor scanning technology, and colocalisation ratio measurement by confocal microscopy.

APPENDIX B OPTIMISATION OF IMMUNOHISTOCHEMICAL METHODS

B.1. Introduction

This appendix complements the data in Chapter 2 and discusses the optimisation of two different methods of AMPA receptor visualisation and quantification using immunohistochemistry: whole-slice visualisation by near-infrared Li-Cor scanning technology and protein colocalisation measurement by confocal microscopy.

Li-Cor immunohistochemistry

Li-Cor near-infrared scanners are marketed for improved Western blot detection and analysis. They remove the need of additional steps after secondary antibody application by the use of near-infrared tagged secondary antibodies, significantly reducing experimental time. Furthermore, the signal wavelength is considered more stable than an enhanced chemiluminescent (ECL) enzymatic detection, which has a variable signal over time. Another convenient trait of this detection method is the ability to label multiple targets on the same blot (or slice), reducing the need to strip or cut membranes. The detection method also allows the detection of faint and strong bands, reducing the risk of saturation. Interestingly, the Li-Cor near-infrared scanner is also marketed for immunohistochemistry use, however, few publications have explored this use as of yet.

The methodology for Li-Cor whole brain slice scanning was based on a recent publication by Eaton et al. (2016), who validated the use of this technique for the quantification of D2 dopamine receptors in the mouse hippocampus, cortex and striatum. They showed that scanning of whole mouse brain slices yielded images of suitable quality for accurate morphometric measurements, which were comparable to measurements taken on a light microscope, showing the images

are of sufficient resolution to recognise the major brain structures. They compared D2 receptor distribution in the cortex, hippocampus and striatum and found that the distribution was comparable to images obtained by confocal microscopy. Furthermore, quantification of the signals in these brain regions was comparable to quantification by Western blot. This study validated the use of near-infrared scanning technology for the quantification of protein signals in whole brain slices. The advantage of using this technology is that it allows the quantification of protein signals in a whole brain region of interest. However, similarly to autoradiography, the signal detected represents the total protein expression, regardless of subcellular location. In order to complement this method, confocal microscopic quantification of protein colocalisation in the dendritic regions of the hippocampus was explored as an adjunct to Li-Cor immunohistochemistry.

Confocal microscopy immunohistochemistry

The method of protein colocalisation using confocal microscopy immunohistochemistry was based on a publication by Zhang et al. (2014), who investigated the bidirectional effects of glycine on NMDA-mediated synaptic plasticity in the CA1 region of the hippocampus in rats. They found that incubation of rat hippocampal slices in low concentrations of glycine (0.6mM) resulted in long term potentiation, as seen as an increase in excitatory postsynaptic currents in the CA1. Using immunohistochemistry and the measurement of NMDA receptor subunit expression, they found that 0.6mM glycine induced an increase in GluN1 NMDA receptor subunits. Conversely, they found that high concentrations of glycine (1.5 mM) resulted in long term depression in the rat hippocampal slices. This also coincided with a decrease in GluN1 and GluN2A expression in the CA1.

This publication measured NMDA receptor subunit expression in the dendritic region of the CA1 by confocal microscopy. They co-stained their slices with antibodies for GluN1 NMDA subunits and PSD95 as their postsynaptic marker, then measured the colocalisation ratios of both proteins, to account for variability in brightness and contrast. This would allow the normalisation and comparison of signals between slices. They also quantified the NMDA receptors by subcellular fractionation and Western blot, and found the confocal colocalisation correlated with the Western blot quantification.

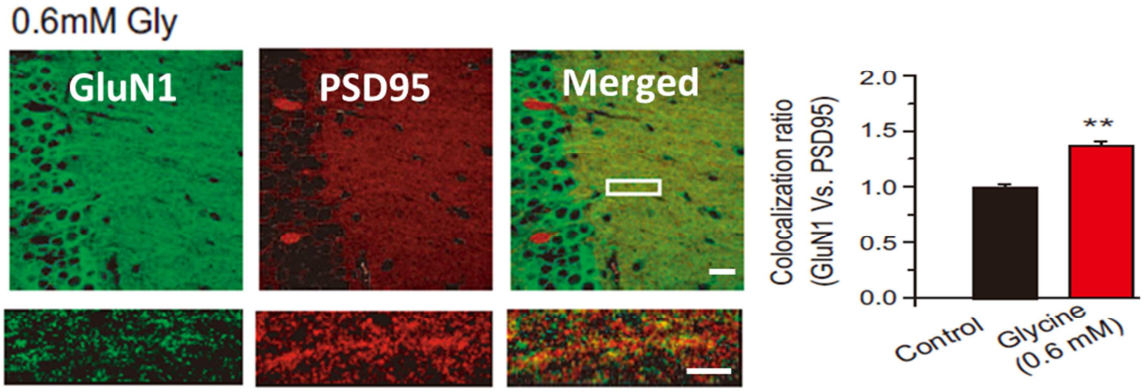


Figure B.1: **Left**: Immunohistochemical labelling of GluN1 (green) and PSD95 (red) and visualisation by confocal microscopy with highlighted dendritic branch (white box). Bar: 20µm. Higher magnification of dendritic branches are shown in the lower panel (bar: 2µm). **Right**: Colocalisation ratio of GluN1 with PSD95 as a normalised measurement of GluN1 expression in dendritic regions. Taken from Zhang et al. (2014).

B.2. Methods

Naïve male Wistar rats (approx. 6-8 weeks old) were intracardially perfused as described in the methodology section of Chapter 2. 24 hours prior to slicing, brains were placed in a 30% sucrose PBS solution. Brains were sliced 40µm thin and placed into 24-well plates for immunolabelling (See Chapter 2, section 2.3.6 for

details). Slices were incubated overnight at 4℃ with primary antibodies: GluA1 (1:1000, rabbit, Millipore), GluA1 phosphoSer845 (1:1000, rabbit, Millipore) and for confocal microscopy, co-labelled with anti-PSD95 (mouse, 1:300, Abcam). Slices were then incubated for 1 hour at room temperature with, for Li-Cor immunohistochemistry: donkey anti-rabbit (1:500, Li-Cor) or goat anti-rabbit (Alexa Fluor568, Invitrogen) and goat anti-mouse (Alexa Fluor 488, Invitrogen). Slices were then mounted on slides with DAPI counterstain.

For Li-Cor immunohistochemistry, slices were scanned using a Li-Cor Odyssey Clx infrared scanner at 21µm resolution. Images were acquired and analysed using Li-Cor ImageStudio version 4.0.

For confocal microscopy, images were acquired with Zeiss Zen Black, then analysed with ImageJ (Fiji).

B.3. Results

Li-Cor

Quantifying antibody signals

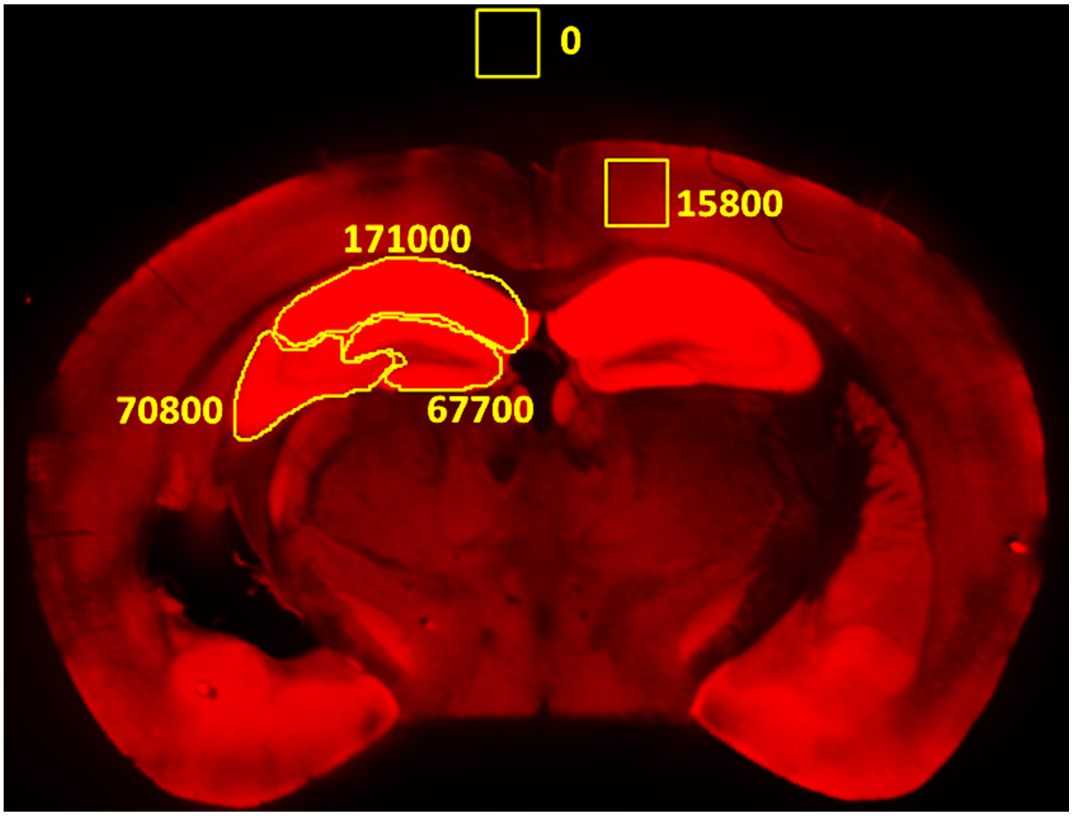


Figure B.2: Representative method of quantification of GluA1 signal intensity in the dorsal hippocampus. The top yellow box shows the background set to zero. The CA1/CA2 (171000), CA3 (70800) and dentate gyrus (67700) regions of the hippocampus are highlighted with their signal intensities (arbitrary units). An area of the cortex is also highlighted with its signal intensity (15800).

Different methods of quantification were trialled, but the optimal settings described below were determined. Figure B.2 above shows a representative image of the quantification method used. An area outside of the brain slice was set as the background, where the signal would be 0. All other signal intensities measured were relative to this background. The brain region of interest was highlighted by

using the freehand analysis tool. This would allow the selection of the entire regions of the hippocampus for signal measurement. The signal measured in a particular area was divided by the size of the shape drawn (in pixels) around the area. This allowed the normalisation of signals between brain slices, where the region of interest could vary in size. All signals were therefore given as signal/area. The antibodies were then titrated to determine the correct concentration for immunolabelling.

Antibody titration

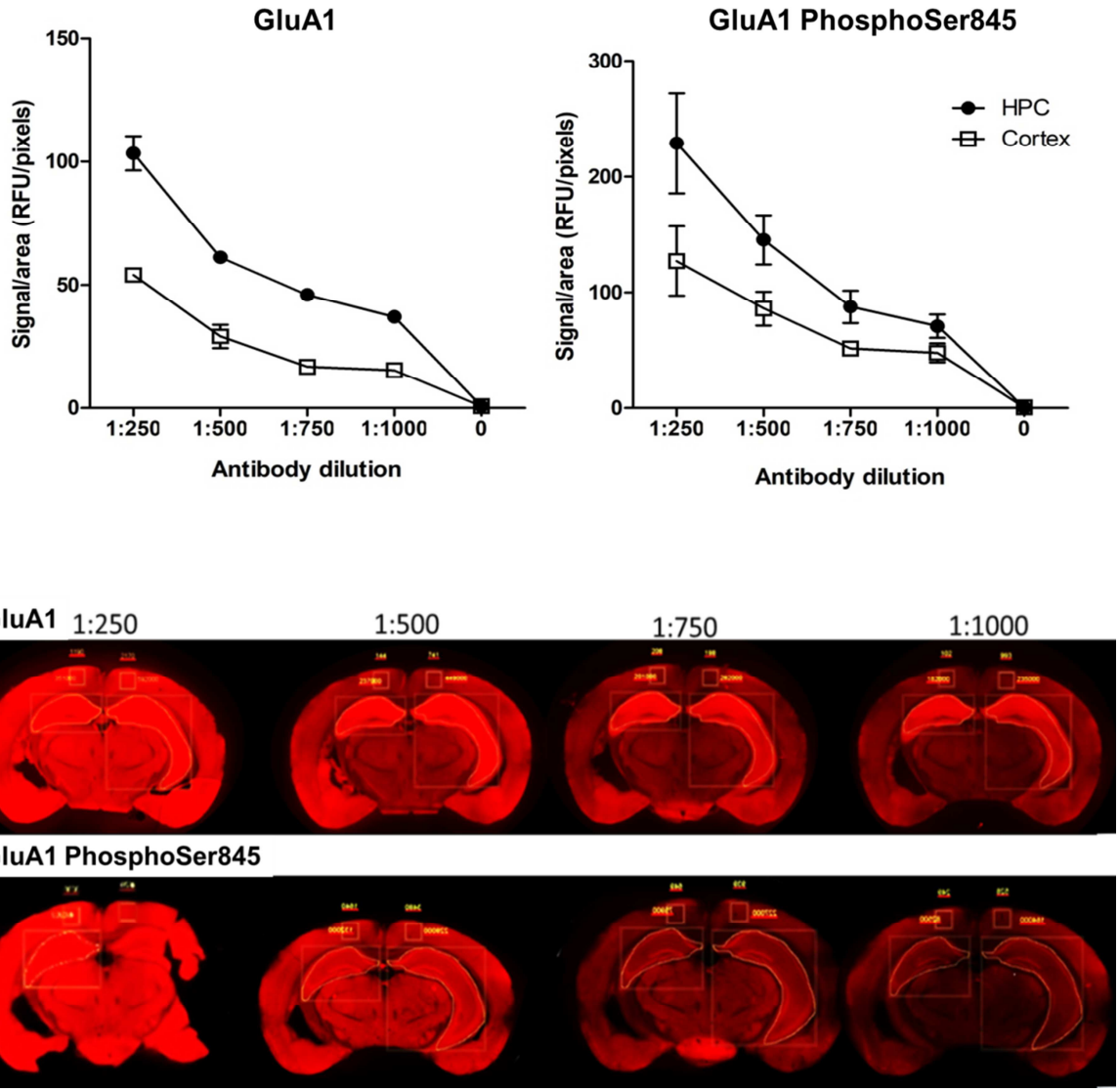


Figure B.3: **Top**: Quantification of GluA1 (left) and GluA1 phosphoSer845 (right) antibody titration signals. Data points are means±SEM, n=4 per titration. **Bottom**: Representative scans of brain slices for the corresponding antibody titration.

Titration of the antibodies showed a near linear relationship between the antibody concentration and the signal intensity (Figure B.3). As previously mentioned, the signals detected during scanning are absolute values; therefore they could not be altered by adjusting the brightness and contrast settings. All slices were scanned

using the same parameters; therefore they were deemed to be comparable. The linear relationship of antibody dilution to signal intensity allowed the selection of any dilution so 1:1000 dilution was selected for future experimentation as the signal intensity was detectable but low enough to potentially detect changes in AMPA receptor expression without saturating the scanner. This also made experiments more economical as less antibody was required.

Slice orientation

When slicing coronal sections of the brain, there was some variability in the level of the hippocampus due to not slicing at an exactly straight angle, and often, slices were obtained with one half of the brain showing the dorsal hippocampus, and the other with the ventral hippocampus (as shown in Figure B.4). As the ventral hippocampus was the region of interest in this study, it was important to differentiate between the dorsal and ventral hippocampus clearly. In order to achieve this, horizontal sections of the brain were performed, as in this orientation; ventral and dorsal levels of the hippocampus are distinguishable.

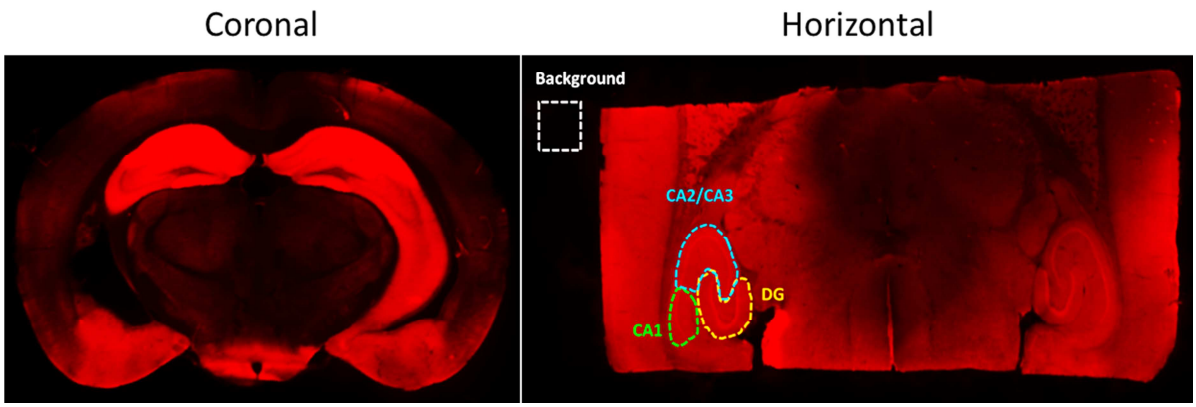


Figure B.4: Brain slice sectioning orientation to obtain the ventral hippocampus. **Left**: coronal sectioning of the hippocampus often resulted in both dorsal and ventral hippocampus sections due to crooked slicing. **Right**: Horizontal sections resulted in much clearer and distinct ventral hippocampus slices. DG: dentate gyrus.

Background

In order to remove any non-specific background signals from the secondary antibody, a control slice was run alongside stained slices where they were incubated in the absence of the primary antibody and then probed with the secondary antibody. The corresponding regions of interest (ROI) were highlighted, and then the signal was divided by the area of the drawn region. This value was then subtracted from the signals previously measured.

The formula for signal intensity measurement was therefore as follows:

$$AMPA\ signal = \frac{signal\ in\ ROI}{area} - \frac{background\ signal\ in\ ROI}{area}$$

Subcellular localisation of phosphorylated GluA1

An interesting finding while optimising Li-Cor immunohistochemistry experiments was the difference in distribution of the phosphorylated and total GluA1 subunits in the hippocampus. This was clearly visible in brain slices scanned on the Li-Cor scanner, as shown in **Figure B.6**A. In all slices, it appeared that GluA1 staining was concentrated in the dendritic regions of the hippocampus (Figure B.5A), whereas phosphorylated GluA1 appeared concentrated in the somatic region (Figure B.6A). This difference in distribution was further investigated using confocal fluorescence microscopy, where slices were stained for GluA1 or the phosphorylated GluA1 (Ser845), with βIII tubulin to act as an intracellular marker. Results also showed a clear difference in distribution.

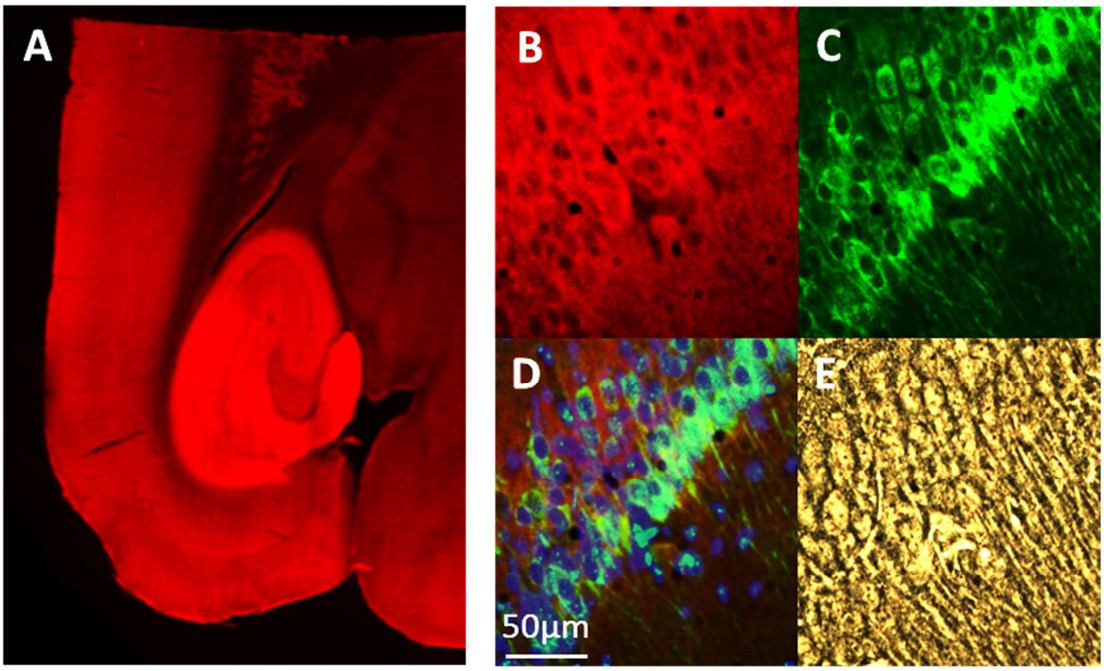


Figure B.5: Distribution of GluA1 AMPA receptor subunits visualised by Li-Cor near-infrared scanning technology **(A)** and confocal fluorescence microscopy **(B-E)**. **B**: GluA1, **C**: βIII tubulin, **D**: merged with DAPI, **E**: brightfield view.

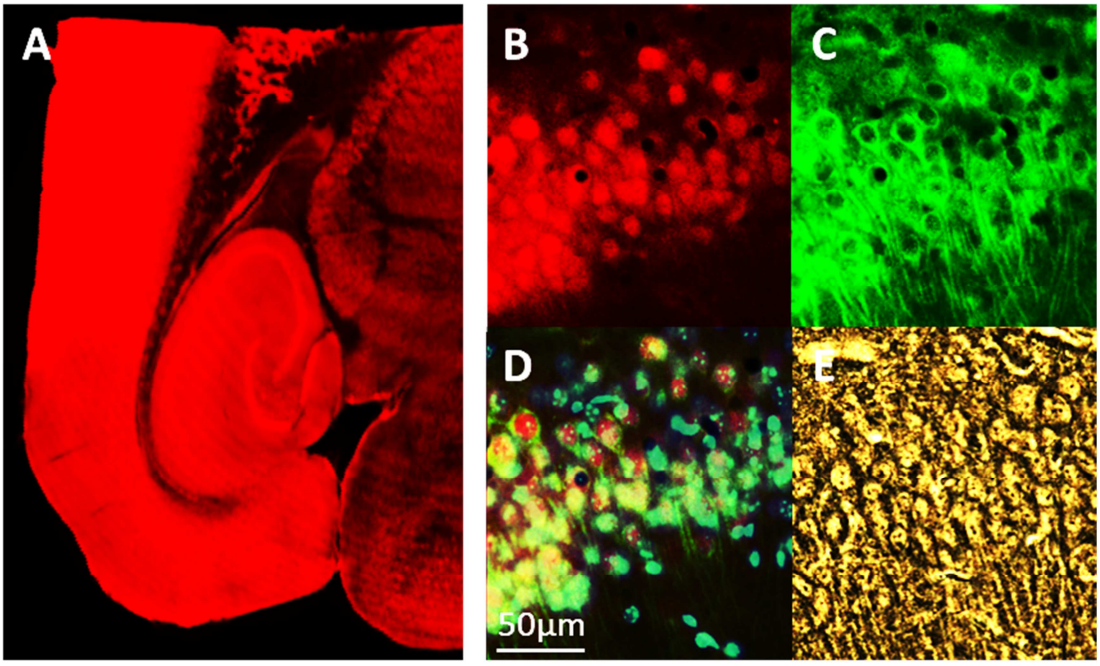


Figure B.6: Distribution of GluA1 phosphoSer845 AMPA receptor subunits visualised by Li-Cor near-infrared scanning technology **(A)** and confocal fluorescence microscopy **(B-E)**. **B**: GluA1 phosphoSer845, **C**: βIII tubulin, **D**: merged with DAPI, **E**: brightfield view.

Analysis of GluA1 staining by confocal fluorescence microscopy showed that GluA1 was present in the cell bodies and dendrites, (Figure B.5B-D). Analysis of the phosphorylated GluA1 subunits showed a distinct grouping in the somatic region of the hippocampus (Figure B.6A). Higher resolution imaging of the CA1 showed that they were located on the soma, and this distribution was again different to the total GluA1 seen in Figure B.5B. It was not possible to determine whether phosphorylated GluA1 was present intracellularly or at the cell surface, therefore a Z stack was performed using confocal microscopy to address this question. The Z stack produced a 3-dimensional image by scanning multiple layers of the somatic region. Interestingly the Z stack revealed that the phosphorylated GluA1 subunits appeared to be expressed both intracellularly and at the cell surface.

Confocal Microscopy

One of the limitations of using autoradiography was that it only showed the total amount of AMPA present in a brain slice, without accounting for intracellular stores. Visualisation by the Li-Cor near-infrared scanner did not address this issue, as the whole brain slice was scanned and the resolution was not high enough ensure the capture of dendritic regions alone. The method of measuring colocalisation ratios in dendritic branches would therefore allow the quantification of synaptic proteins in the absence of intracellular stores (Zhang et al., 2014), which could then complement the data obtained from Li-Cor whole brain slice imaging.

Antibody titration

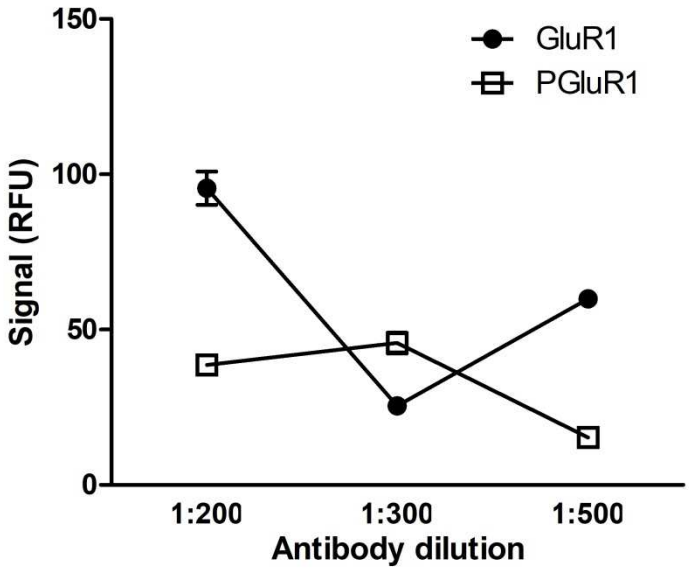


Figure B.7: Antibody titration for confocal microscopy. Data are means±SEM. n=3 per antibody dilution.

The antibody titration, unlike the Li-Cor near-infrared antibodies, showed a non-linear relationship of the antibody dilution with the signal intensity. A 1 in 1000 titration was also attempted but the signal was not detectable on the confocal microscope (data not shown). For the purpose of this study and to conserve antibody stocks, the titration of 1:500 was selected so the signal was detectable, but low enough to detect any potential changes in AMPA receptor expression.

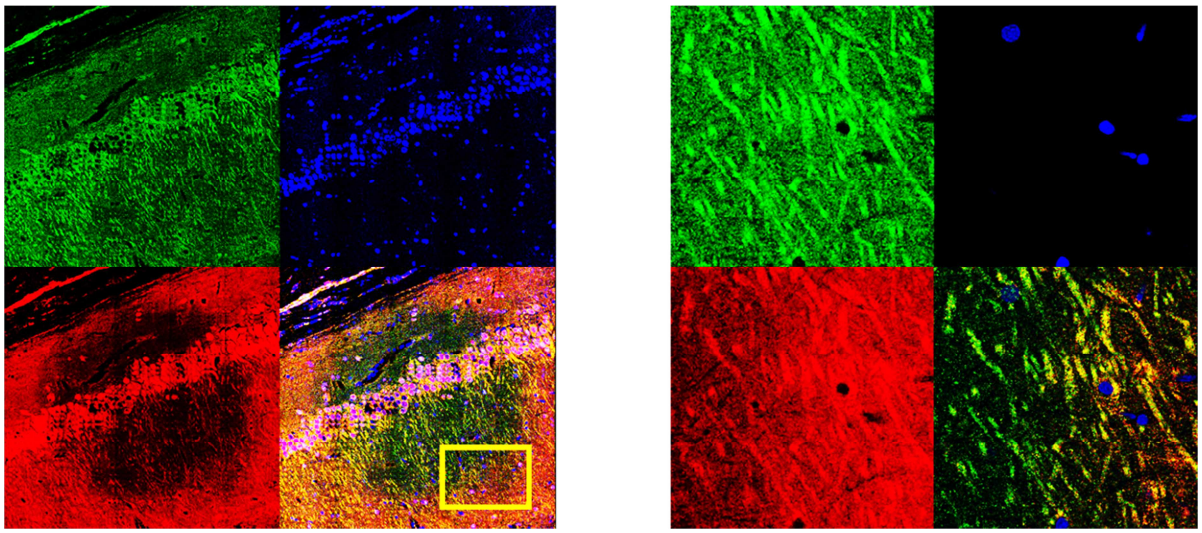


Figure B.8: Left: Representative confocal tile scan of the CA1 region of the ventral hippocampus image with a representative region of interest highlighted (yellow box) for quantification in the dendritic layer. **Right**: Region of interest 63x magnified for quantification of protein colocalisation. Green (top left): PSD95, red (bottom left): GluA1 PhosphoSer845, blue (top right): DAPI, bottom right images are merged channels

Figure B.8 shows representative confocal images in the CA1 region of the hippocampus. The cell body region was visible, and importantly, the dendritic layer was clearly distinguishable. This would allow the selection of dendritic regions for the quantification of synaptic proteins.

Quantification

The Pearson's correlation coefficient (PCC) was used as a measure of colocalisation as this metric includes normalisation. This would allow for the variation in intensity coming from the difference in acquisition settings such as the depth focus, brightness, contrast, gain offset and background. The normalised values of colocalisation therefore ranged from 1, signifying complete correlation; to -1, demonstrating complete anti-correlation (Dunn et al., 2011).

The dendritic layers of the hippocampus were clearly visible in brain slices (Figure B.8), and DAPI staining was used in order to select a region lacking cell bodies. The region to analyse was highlighted and analysed for colocalisation using the coloc2 plugin in ImageJ. An example of the analysis is shown in **Figure B.9** below.

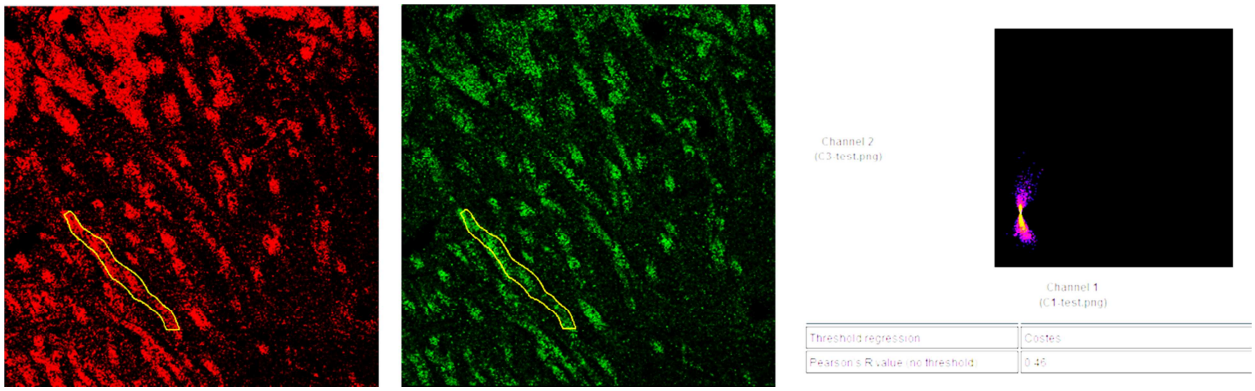


Figure B.9: Representative image demonstrating the colocalisation analysis on the dendritic branches of the hippocampus. One dendritic branch is highlighted in yellow. Red: GluA1 phosphoSer845, green: PSD95. Right: analysis output from ImageJ showing the Pearson's correlation coefficient (R value)

As the baseline correlation of GluA1 or GluA1 phosphoSer845 with PSD95 in control rats was neither 1 nor -1 (see Chapter 2, section 2.4.6), this method would be able to detect any changes in the colocalisation of AMPA receptor subunits with

PSD95, translated as the changes in insertion or removal of AMPA receptors in the postsynaptic membranes.

B.3. Conclusions

Whole brain slice immunohistochemistry and visualisation by the Li-Cor near-infrared scanner is an interesting method of protein detection and quantification at a whole tissue level. The hypothesis was that the results from this method would mirror those obtained by Wright et al. (2018), but at a subunit-specific level. The drawback of this method however, similarly to autoradiography, is that it does not differentiate between somatic and synaptic proteins. This is the reason the visualisation by confocal microscopy was introduced, as the colocalisation of AMPA receptors with the postsynaptic marker PSD95 would allow the measurement of synaptic proteins in the dendritic layers of the hippocampus. The hypothesis was that using the higher resolution confocal microscopy would complement and add further information to the data obtained by whole brain slice immunohistochemistry.

These methods were both applied on the rat brains from the four reinstatement groups of the heroin CPP described in Chapter 2, to explore the effects of MLA on the expression of postsynaptic AMPA receptors in the ventral hippocampus.

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